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Biochemical and genetic characterization of brochocin-C, a bacteriocin produced by *Brochothrix campestris* ATCC 43754.

by

Alison Poon

A thesis submitted to
the Faculty of Graduate Studies and Research in partial fulfillment of
the requirements for the degree of Master of Science

in

Food Microbiology

Department of Food Science and Nutrition

Edmonton, Alberta

Fall 1995

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Biochemical and Genetic characterization of brochocin-C, a bacteriocin produced by *Brochothrix campestris* ATCC 43754 submitted by Alison Poon in partial fulfillment of the requirements for the degree of Master of Science in Food Microbiology.

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29 September 1995

Abstract

Brochocin-C is a strongly hydrophobic bacteriocin produced by *Brochothrix campestris* ATCC 43754 that is active against a broad spectrum of Gram-positive bacteria. Crude brochocin-C was thermostable up to 121°C for 15 min, pH stable from 2 to 9, and inactivated by proteolytic enzymes. The bacteriocin was purified, its amino acid sequence (aa) determined, and a site-specific 23-mer oligonucleotide probe hybridized to a 4.2-kb *Eco*RI genomic DNA fragment. Nucleotide sequencing revealed two open reading frames (ORFs). The bacteriocin structural gene (ORF1), encodes a 77 aa prepeptide and the immunity gene (ORF2), encodes a 60 aa immunity protein. Cleavage of the prepeptide occurs after a double glycine motif to yield a 59 aa mature bacteriocin and an 18 aa leader peptide. This leader peptide bears significant homology to leader peptides of the class II bacteriocins of lactic acid bacteria. The molecular weight of the purified compound was determined by mass spectrometry to be 5241.21 ± 1.22.

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1. General Introduction

Lactic acid bacteria (LAB) have long been considered as safe bacteria for consumption in foods. They have traditionally been used to preserve and enhance the safety of foods due to their antagonistic activity towards many food spoilage and foodborne pathogenic microorganisms. The biopreservative capacity of LAB can be attributed to production of organic acids (lactic and acetic acids) which lower the pH of the food, and to production of inhibitory substances such as hydrogen peroxide, diacetyl, and bacteriocins.

Bacteriocins are biologically active proteins that form a heterogeneous group with respect to the producing organism, antimicrobial spectrum, molecular size, physical and chemical properties, stability, mode of action, and genetic determinants. In recent years LAB, or the bacteriocins that they produce, have been looked upon as biological preservatives to replace the use of chemical additives currently used in foods. The proteinaceous nature of bacteriocins makes them sensitive to an array of proteolytic enzymes, including those of gastric and pancreatic origin, which implies that their ingestion should not affect the microbial flora of the gastrointestinal tract. While most bacteriocins have a relatively narrow spectrum of activity that is inhibitory to only closely related species, others have a relatively broad spectrum of activity. Nisin, produced by *Lactococcus lactis* subsp. *lactis*, has a broad spectrum of activity that includes many Gram-positive bacteria and inhibits the germination of *Clostridium* and *Bacillus* spores.

Meat provides an excellent medium for growth of spoilage and pathogenic

microorganisms. However, bacteriocins discovered thus far have had limited success in biopreservation of meat. Nisin is approved as a food additive in various products in over 45 countries, but has limited effectiveness in meats. It is poorly soluble above pH 5.0, interacts strongly with phospholipids (Henning *et al.*, 1986), is not active against all spoilage or pathogenic microorganisms associated with meat, and the producing organism does not grow well in chilled meats (Hastings and Stiles, 1991). Pediocin PA-1, produced by *Pediococcus acidilactici* PAC 1.0, does not appear to break down in meat but it has a narrow spectrum of activity that is limited to closely related LAB and to *Listeria monocytogenes*. This bacteriocin has been shown to be effective in lowering *L. monocytogenes* counts by 0.5 to 2.2 log cycles in inoculated meat, but this effect depends on the concentration of the bacteriocin (Nielsen *et al.*, 1990). Limitations to use of bacteriocins discovered thus far in a meat system opens the area to further research into ways to prevent the inactivation of the bacteriocin by proteases inherent in the meat system, discovery of broad spectrum bacteriocins that effectively target meat spoilage and pathogenic microorganisms, and discovery of bacteriocins able to retain their biological activity within the normal pH range of meat.

An antagonistic substance produced by *Brochothrix campestris* ATCC 43754 was first reported by Siragusa and Nettles Cutter (1993) and termed a bacteriocin (brochocin-C) based on its sensitivity to proteolytic enzymes, inhibitory spectrum, catalase insensitivity, and heat stability. However, the antimicrobial substance was

not further characterized to determine its primary (amino acid) structure and was not shown to be a bacteriocin similar to nor different from previously characterized LAB bacteriocins. The relatively broad spectrum of brochocin-C includes inhibition of *B. thermosphacta*, an important spoilage agent of meats, and warrants further investigation to the nature of its biological activity.

The objective of this study was to determine the biochemical and genetic characteristics of brochocin-C and to determine whether it has similarities to well-characterized bacteriocins of LAB. Purification of the antimicrobial substance, determination of the amino acid sequence, and the deoxyribonucleic acid (DNA) sequence coding for the peptide will be reported. While neither *B. thermosphacta* nor *B. campestris* has been reported to be pathogenic, the latter has been isolated from soil but not from food sources. Consequently, to have potential for application in a food system, the gene encoding the bacteriocin peptide will be introduced via a suitable vector into a heterologous food-grade organism to obtain brochocin-C expression.

2. Literature review

2.1. THE ASSOCIATION OF *BROCHOTHRIX THERMOSPHACTA* WITH SPOILAGE OF MEAT AND MEAT PRODUCTS.

2.1.1 Introduction

Meat and most meat products are highly susceptible to microbial spoilage and growth of foodborne pathogens. The exterior of animals is soiled with microorganisms acquired on the farm or during transportation to the slaughter house. The amount of microbial contamination transferred to carcasses varies and it is a reflection of the sanitation practised during slaughter, skinning, and opening of the body cavity. Direct or indirect contamination with the animal's hide, legs, or hooves, with gut contents, faecal material, or contaminated equipment all result in transfer of microorganisms that lead either to spoilage of the meat, or to contamination of the meat with microorganisms that are capable of causing illness. In Canada, it is estimated that the meat industry sustains annual losses of 200 million dollars due to spoilage. Foodborne illnesses related to meat consumption result in annual losses of 500 million dollars due to product recalls, loss of work productivity, legal costs, and medical expenses (Greer, 1993). Consequently, the control of bacteria associated with meat spoilage and pathogenicity has significant economic consequences.

Factors that affect the storage life of meat and the progress of spoilage development by microorganisms include the storage temperature, initial microbial load, and gaseous environment (Fig. 1). Proper chilling of carcass meat selects for

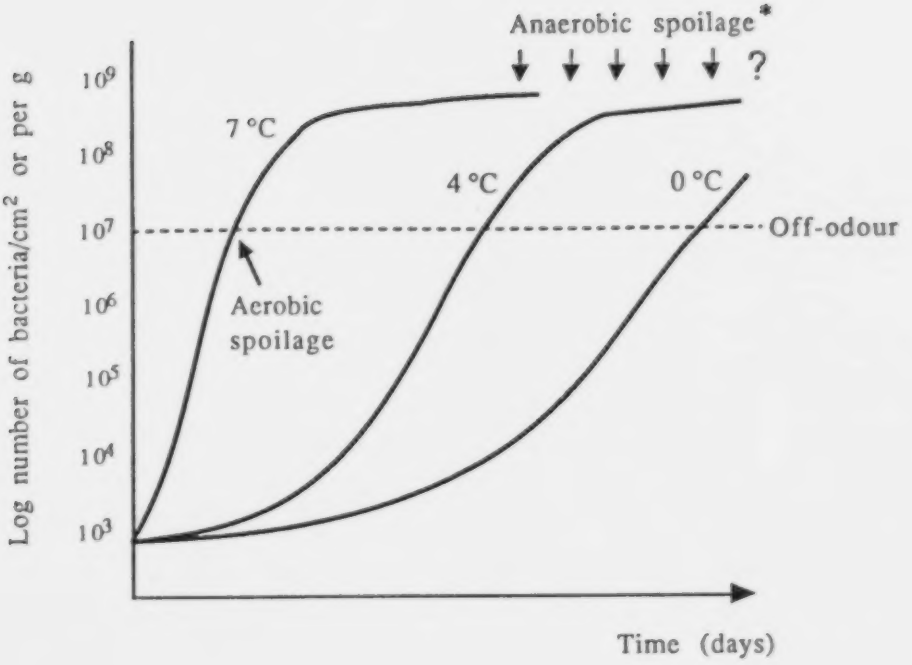

Figure 1. A schematic comparison of the aerobic and anaerobic spoilage of meat at different storage temperatures (reprinted from Stiles, 1991 with permission).

* Time at which spoilage occurs under anaerobic conditions with lactic acid bacteria dominating the microflora cannot be accurately predicted.

growth of psychrotrophic strains of microorganisms (Stiles, 1991). The storage temperature directly affects the time required for the psychrotrophic microorganisms to grow to numbers that cause spoilage. Organisms able to grow at low temperatures do so at a faster rate at 7°C than at 0°C. Therefore, to maximize the storage life of meat, the optimal storage temperature for fresh meat is -1.5°C because this retards the growth of most psychrotrophs yet it does not freeze the meat. Raw meat can be exposed to considerable variations in temperature, ranging from 0 to 10°C during storage, transport, processing, and retail display (Greer, 1993).

There are five main groups of organisms that contribute to various forms of meat spoilage, these include: pseudomonads, enterobacteria, lactobacilli, *Brochothrix thermosphacta*, and *Shewanella putrefaciens* (Gill and Molin, 1991). Of these, the first three are inevitable contaminants of nonsterile meats, whereas the latter two can be minimized with attention to proper hygiene. Therefore, to extend the storage life, the goal would be to avoid contamination with organisms that result from improper hygiene, and to keep the initial level of indigenous microoganisms as low as possible because these must grow to high population levels before spoilage occurs.

The gaseous environment of meat storage has a marked effect on the microbiology of the meat. Under aerobic chilled storage, growth of Gram-negative rod-shaped bacteria such as pseudomonads is favoured. The normal pH (5.6 to 5.8) of fresh meat prevents growth of some spoilage bacteria, while the faster

growth rate of pseudomonads establishes them as the dominant microflora (Shelef, 1981). *Pseudomonas* spp., in particular *P. fragi*, will predominate and cause putrefactive spoilage odours from amino acid metabolism (Greer, 1993). In general, off-odours are detected at 10^7 to 10^8 colony-forming units (cfu) per g or cm^2 of meat, discoloration at 10^6 cfu/g or cm^2 and as sliminess at 10^8 to 10^9 cfu/g or cm^2 (Stiles, 1994). In contrast, when meat is stored in gas impermeable plastic under anaerobic conditions, such as under vacuum or packaged in a gaseous environment with elevated levels of carbon dioxide (CO_2), growth of aerobic spoilage organisms (e.g. moulds and pseudomonads) is inhibited, while the growth of yeasts, lactic acid bacteria (LAB), and *B. thermosphacta* is not affected (Gill and Molin, 1991; Stiles and Hastings, 1991). Carbon dioxide is a bacteriostatic agent that acts by extending the lag phase and slowing down the growth rate of sensitive microorganisms. As shown by the graph in Fig. 1, the LAB do not produce off-flavours or off-odours at 0 or 5°C in the same time frame as the aerobic spoilage microflora. Spoilage occurs at some time after maximum population of 10^8 to 10^9 cfu/g or cm^2 is reached (Stiles, 1994). The change in the microflora between aerobically and anaerobically packaged meat is responsible for the extended storage life of vacuum and modified atmosphere packaged meat with elevated levels of CO_2, but this is dependent on storage at ≤5°C.

2.1.2 The association of *B. thermosphacta* with spoilage of meat.

Brochothrix thermosphacta is a Gram-positive, facultative anaerobe that is

an important spoilage organism of meat and meat products stored at chill temperatures, because it grows well on meat and imparts offensive off-odours and off-flavours under aerobic conditions, and in some circumstances, in vacuum or modified atmosphere packaged meats (Gardner, 1981). Conditions that prevail during such storage include low temperature, decreased oxygen (O_2) and increased CO_2 concentrations. Other factors that inhibit or enhance the growth of *B. thermosphacta* on meats during storage include the chemical and physical properties of the meat; storage temperature; pH; gaseous environment; presence of salt, sodium nitrite, or sulphur dioxide; and interactions with other spoilage bacteria.

Brochothrix thermosphacta was originally isolated from chill stored, fresh pork sausage meat by Sulzbacher and McLean (1951) and it was described as *Microbacterium thermosphactum*. These workers were among the first to recognize the importance of this organism in meat bacteriology. It comprised a large proportion of the meat microflora and was responsible for the development of souring. Based on differences in cell morphology (Davidson *et al.*, 1968; Jones, 1975), enzymology and protein profiles (Collins-Thompson *et al.*, 1972; Robinson, 1966), peptidoglycan structure (Schleifer, 1970; Schleifer and Kandler, 1972), and in DNA base composition (Collins-Thompson *et al.*, 1972) between *M. thermosphactum* and *M. lacticum*, the prototype species of the genus, Sneath and Jones (1976) proposed that *M. thermosphactum* be classified in a new genus *Brochothrix*. Renamed *B. thermosphacta*, this species remained the only one in

the genus until Talon *et al.* (1988) described a new species, *B. campestris*. As a result of the relatively recent recognition of the latter species, much of what is known about the genus has been derived from studies on *B. thermosphacta*.

Several studies have reported the effects of *B. thermosphacta* on sensory quality of beef, pork, poultry, lamb, and meat products. *B. thermosphacta* is usually found only on the meat surface (Gill and Penney, 1977) and contamination most likely occurs during slaughter and postslaughter procedures.

2.1.2.1 Beef

Ample opportunity exists for fresh beef to become contaminated with *B. thermosphacta*. As already mentioned, a source of contamination could be the hides of slaughtered cattle (Newton *et al.*, 1978), but contamination also arises from soil from abattoirs, air from the chilling room, hands of workers, and contaminated equipment used in boning of the carcass (Gardner, 1981). Under aerobic conditions, pseudomonads will dominate the microflora. However, primal cuts from fresh beef carcasses are often packaged in gas impermeable films for shipping to distant markets. Restriction of O_2 transmission in the packaging film and alteration of the gaseous environment to one with elevated levels of CO_2 creates suitable conditions for growth of lactic acid bacteria and *B. thermosphacta*, both of which are capable of causing spoilage of beef. The extent of growth of *B. thermosphacta* on vacuum packaged fresh beef depends on the pH of the muscle, the amount of surface fat, and the availability of O_2 (Egan and Grau, 1981). Under aerobic conditions, pH has little effect on inhibition of growth of the organism;

however, under anaerobic conditions, normal pH meat (pH<5.8) inhibits the growth of *B. thermosphacta* due to the presence of lactic acid. *B. thermosphacta* shows an increased sensitivity to lactic acid under anaerobic conditions (Egan and Grau, 1981) but not under aerobic conditions. This may explain the fact that the organism grows better in an aerobic system than an anaerobic system. Lean muscle under restricted O_2 conditions also does not support growth of the organism, whereas fat surfaces can support growth of high numbers of the organism (10^7 cfu/cm^2) because of the high pH (6.8 to 7.0). Growth rate on adipose tissue is similar to that on muscle but growth ceases at approximately 10^8 cfu/cm^2 instead of one log unit higher. Glucose is present in the fatty tissue but in lower amount than in muscle. The low availability of carbohydrate substrates in combination with the high pH of adipose tissue leads to faster spoilage due to degradation of amino acids beginning at 10^4 cfu/cm^2 and production of associated off-odours which are detected at 10^6 cfu/cm^2 (Nottingham *et al.*, 1981). Enzymes necessary for hydrolysis of proteins are normally suppressed until the late logarithmic phase of growth in the presence of readily utilized carbohydrate substrates. In studies on the spoilage of vacuum packaged beef of normal pH, an initial inoculum of 10^4 cfu/cm^2 grew to 10^6 cfu/cm^2 after 10 days of storage at 5°C in film with low O_2 permeability, remained at 10^5 to 10^6 cfu/cm^2 for up to 35 days of storage, and the beef remained sensorily acceptable (Egan and Grau, 1981). However, when packaged in film with high O_2 permeability, levels of 10^8 cfu/cm^2 were reached in 10 days of storage at 5°C. This was associated with off-odours and

progressively unacceptable meat samples. The permeability of the films commonly used in commercial practice prevent populations of *B. thermosphacta* reaching $>10^6/cm^2$, unless the pH of the beef is >5.9 (Egan and Grau, 1981).

2.1.2.2 Pork

B. thermosphacta can be regarded as a common contaminant of pork, and under certain conditions, it is important in the spoilage of pork. One study reports that *B. thermosphacta* grows equally well on refrigerated pork stored aerobically or vacuum packaged, and the pH (normal or high) of the pork had no effect on the growth rate of the organisms (Hermansen, 1980). Pale, soft, and exudative (PSE) pork has a lower pH than normal pork, whereas dark, firm, and dry (DFD) pork has a higher pH. It has been shown that under aerobic conditions, *B. thermosphacta* grows best on DFD pork and least on PSE pork due to a longer lag phase in the latter (Greer and Murray, 1988). Pork cuts stored under vacuum in packaging films with low O_2 transmission rates were grossly spoiled by the growth of *B. thermosphacta* after 2 weeks of storage at 4.4°C and after 5 weeks of storage at -1.5°C. (McMullen and Stiles, 1991). With pork packaged in CO_2 and stored at -1.5°C, storage life was extended to 18 to 26 weeks (McMullen and Stiles, 1991).

2.1.2.3 Lamb

B. thermosphacta has been isolated from lamb chops stored in an O_2 permeable film and in vacuum packages (Barlow and Kitchell, 1966). The extra handling during dressing of carcasses was cited as the reason for a higher

frequency of isolation of the organism from lamb than from beef (Newton *et al.*, 1977). In addition, lamb generally has a higher pH, possibly due to excessive fatty tissue and this may affect growth.

2.1.2.4 Vacuum packaged meat products

In prepacked meats, *B. thermosphacta* grows well at the meat - plastic film interface (Ingram and Dainty, 1971). In meat products such as vacuum packaged sliced cooked ham, *B. thermosphacta* often constitutes a significant proportion of the microflora (Qvist and Mukherji, 1981). Such products and their storage conditions select for growth of LAB and *B. thermosphacta*, and the organoleptic spoilage of vacuum packaged luncheon meats is determined by population levels of each of these organisms. Under vacuum, LAB become dominant. Although they spoil meat products, LAB do so at a much slower rate and at much higher population levels than *B. thermosphacta*. One study reported that levels of 1.5 to 2.0 x 10^7 cfu of *B. thermosphacta* per gram of sliced cooked ham made the product organoleptically unacceptable, whereas the same level of LAB produced no undesirable changes (Qvist and Mukherji, 1981). *B. thermosphacta* is associated with off-odours and off-flavours if present in sufficient numbers and thus, causes a more rapid and pungent spoilage of luncheon meats than the LAB. The presence of *B. thermosphacta* is an indication of post-heating contamination. The organism is not heat resistant and should not survive adequate heat processing. On a commercial cleaned production line, 10^2 to 10^3 *B. thermosphacta* per cm^3 could be found on swabs taken from tables, walls, and

floors (Qvist and Mukherji, 1981). Special care should be taken to avoid cross contamination by applying good manufacturing practices.

2.1.3 Factors affecting the growth of *B. thermosphacta* on meats.

When populations of *B. thermosphacta* reach levels of $\geq 10^6$ cfu/g, spoilage can be detected. However, some environmental conditions, such as oxygen, pH, and temperature can prevent numbers from reaching such levels. In addition, curing additives may have an effect.

2.1.3.1 Salt (NaCl), sodium nitrite ($NaNO_2$) and temperature

Cooked, cured meats such as sliced ham contain 2 to 4% NaCl, 20 to 100 ppm $NaNO_2$, have a pH 5.5 to 6.5, and they are stored at refrigeration temperatures (5°C). This would not likely prevent growth of *B. thermosphacta*, assuming that *in vitro* tests mimic the *in vivo* situation on meat. Different workers have shown that the organism tolerates salt levels up to 10% (w/v) (Talon *et al.*, 1988), it grows in APT broth with 25 to 50 μg $NaNO_2$/mL (Collins-Thompson and Rodriguez Lopez, 1980), and it grows at temperatures ranging from 0 to 30°C (Gardner, 1981; Sneath and Jones, 1976; Jones, 1992). Growth of *B. thermosphacta* can be inhibited by nitrite but the degree of inhibition is related to the pH of the medium and temperature of incubation. The inhibitory effect is increased with low pH, low temperature, and high concentrations of nitrite (Brownlie, 1966).

2.1.3.2 Gaseous environments

Although *B. thermosphacta* grows better under aerobic than anaerobic

conditions, it is well documented that the organism is able to grow on vacuum packaged meats (Egan and Grau, 1981; Qvist and Mukherji, 1981). Inhibition of *B. thermosphacta* only occurs in environments where the O_2 is below 0.2% (Gardner, 1981). A 20% concentration of CO_2 inhibits the growth of most aerobic meat spoilage organisms but it has been shown to have little effect on the growth of *B. thermosphacta* (Roth and Clark, 1972).

2.1.3.3. pH

The optimum pH for growth of *B. thermosphacta* is pH 7.0, but growth occurs within the range of 5 to 9 (Brownlie, 1966). The low pH of normal meat (5.6 to 5.8) arises from production of lactic acid from muscle glycogen during the onset of *rigor mortis*. High pH (>6.0) meat results when the animal experiences preslaughter stress, which causes the muscle glycogen to be depleted before death and less lactic acid being produced post-mortem. High pH meat is commonly referred to as dark, firm, dry (DFD) meat, and it spoils more rapidly than normal pH meat. DFD is devoid of preferred carbohydrate (glucose), so amino acids are used by the spoilage organisms as an energy source without delay. This results in production of ammonia and sulphur-containing compounds as soon as bacterial growth begins (Newton and Gill, 1978). It has been suggested that growth of *B. thermosphacta* under anaerobic conditions depends largely on the pH of the meat (Egan and Grau, 1981). With normal pH meat, growth of *B. thermosphacta* is not observed even after 30 days of chilled storage. Under similar conditions with the pH 6.0 to 6.4, growth is only slightly retarded and the population grows to $>10^7$

cfu/g within 8 d. Whereas with both normal and high pH mucscle under aerobic conditions, *B. thermosphacta* grows rapidly and reaches cell densities of 10^9 cfu/g within 9 to 10 days of storage at 5°C.

2.1.3.4 Substrates

Meat contains a large number of low molecular weight soluble components in particular, glucose and amino acids, which bacteria use for growth. These substrates are utilized and depleted before proteolysis of muscle proteins (Gill and Newton, 1978). Bacteria such as *B. thermosphacta* can grow on the meat surface where glucose concentrations are low. With increased cell density, the rate of glucose utilization is greater than its rate of replacement by diffusion from within the meat (Nottingham *et al.*, 1981). At 10^8 cfu/cm^2, the level at which spoilage is detectable, surface glucose levels have been exhausted and the ammonia concentration rises which is indicative of degradation of amino acids.

Under anaerobic conditions, *B. thermosphacta* utilizes only a few substrates (glucose and ribose) and produces primarily lactic acid as its end product. These substrates become depleted when the cell density is approximately 10^8 cfu/cm^2, so growth stops due to nutrient limitation (Nottingham *et al.*, 1981). With high pH meat (pH>6.4) little if any glucose is available (Grau, 1988, Nottingham *et al.*, 1981), so ribose probably supports anaerobic growth. Consequently, better growth of *B. thermosphacta* would be expected on high pH meat than on normal pH meat under anaerobic conditions, partly because the rate of inosine-monophosphate and inosine degradation to produce ribose is faster in higher pH

meat.

Under aerobic conditions, a range of substrates is available and not all are exhausted when growth stops so the limiting factor appears to be the oxygen supply (Nottingham *et al.*, 1981). Glucose, ribose, and glycerol are used preferentially by *B. thermosphacta* as substrates when growing on meat (Grau, 1988) with acetic acid and acetoin being produced as end products (Grau, 1988). These compounds, or their derivatives, produce the 'dairy' off-odours that characterize the growth of *B. thermosphacta*. Short branched acids (*iso*butyric and *iso*valeric acids) can be produced from leucine and valine, respectively, and can also contribute to spoilage in aerobically stored fresh meats (Dainty and Hibbard, 1980). Glycerol-3-phosphate and inosine can also serve as substrates but they are used only after the substrates listed above have been depleted because they support slow growth of the organism (Grau, 1988).

2.1.3.5 Interactions with other bacteria

An important feature in the ecological importance of *B. thermosphacta* is its interrelationship with other spoilage bacteria (Roth and Clark, 1972). In vacuum packaged beef, *B. thermosphacta* is a poor competitor with the natural microflora, but it grows well in the absence of other bacteria. In competition with large numbers of pseudomonads under aerobic conditions, *B. thermosphacta* grows more slowly and reaches only 5% of the cell density that would be achieved in pure culture because of competition for available O_2 (Roth and Clark, 1972).

Given the association of *B. thermosphacta* with the environment of animals, the elimination of contamination of fresh meats with this organism is highly unlikely. A more suitable approach to control spoilage by this organism would be to implement measures that can restrict or prevent its growth. It has been demonstrated that strict anaerobic conditions, normal pH meat, low temperatures used for storage, the presence of lactic acid, and the presence of a competitive LAB microflora will inhibit the growth of *B. thermosphacta* (Roth and Clark, 1975; Grau, 1980). Problems arise when conditions allow the growth of *B. thermosphacta* to occur, such as increased availability of oxygen and high pH meat (Grau, 1980, 1988; Blickstad and Molin, 1984). These problems can be identified but they are not always avoidable, leading to spoilage problems and the need to look at novel methods to control the growth of the organism when other growth hurdles have been surpassed.

2.2. THE POTENTIAL OF LACTIC ACID BACTERIA AND (OR) THEIR BACTERIOCINS AS PRESERVATIVES.

2.2.1 Introduction

The lactic acid bacteria (LAB) are a group of Gram-positive, nonsporeforming bacteria that produce lactic acid as the main product of carbohydrate metabolism (Kandler, 1983) and include the genera *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*. Different species amongst these genera have been traditionally used as starter cultures for the fermentation of foods and beverages because of

their nonpathogenic nature and for their contribution to flavour and retardation of spoilage. LAB present in the foods contribute to changes in the sensory properties and to its extended shelf life by production of antimicrobial substances such as lactic and acetic acids, hydrogen peroxide, and bacteriocins. Consumer demands for decreased use of chemical additives in foods has spawned research into the use of naturally occurring metabolites to inhibit growth of undesirable bacteria. Natural antimicrobials may replace the use of chemical preservatives such as sulphur dioxide, benzoic, propionic, and sorbic acids, nitrates, and nitrites (Lloyd and Drake, 1975).

2.2.2 Classification of LAB bacteriocins

Bacteriocins, by definition, are antibacterial peptides that are bactericidal against other, usually closely related bacteria (Klaenhammer, 1993). Bacteriocins of LAB can be classified as belonging to one of four distinct classes (Klaenhammer, 1993): (I) small (<5 kDa) membrane active peptides that undergo extensive posttranslational modification and are commonly referred to as lantibiotics [e.g. nisin], (II) small (<10 kDa) heat stable, membrane active peptides commonly referred to as nonlantibiotics [e.g. pediocin PA-1, sakacin A], (III) large (>30 kDa) heat labile proteins [e.g. helveticin J], and (IV) complex bacteriocins comprised of protein plus one or more chemical moieties (lipid, carbohydrate) that is required for biological activity [e.g. pediocin SJ-1]. Bacteriocins are a heterogeneous group of compounds with respect to the producing strain, molecular size, physical and

chemical properties, stability, antimicrobial spectrum, and mode of action. Many of the bacteriocins produced by LAB are only stable at acidic and neutral pH and are inactivated at pH >8.0 (e.g. nisin, pediocin PA-1/AcH, leucocin A-UAL 187).

2.2.2.1 Lantibiotics and nisin

Nisin, produced by *Lactococcus lactis* subsp. *lactis* was the first recognized bacteriocin of LAB and is by far the most thoroughly characterized bacteriocin. Nisin is classified as a lantibiotic because of the lanthionine or ß-methyllanthionine ring structures present in the peptide chain. Several functions that have been postulated for these unusual amino acids include conferring thermal stability to the peptide, crosslinking to retain a stable pore forming conformation, and chemical reactivity important for biological activity (Klaenhammer, 1993). In general, nisin exerts a broader spectrum of antimicrobial action than other LAB bacteriocins. The broad spectrum of nisin is reflected in the fact that the peptide does not require a specific integral membrane receptor for insertion or activity in the susceptible strain (Gao *et al.*, 1991). Nisin is bactericidal to a wide range of Gram-positive bacteria including most LAB, *Staphylococcus aureus*, and *Listeria monocytogenes*, and it prevents the outgrowth of *Bacillus* and *Clostridium* spores. Nisin serves as the prototype LAB bacteriocin because of its broad spectrum of activity and because it is the first LAB bacteriocin to receive GRAS (generally recognized as safe) status in the U.S. by the Food and Drug Administration (FDA). Nisin is licensed for use as a preservative in foods in over 45 countries (Delves-Broughton, 1990). Its sensitivity to α-chymotrypsin, heat stability at low pH, and

nontoxic nature have promoted its widespread use (Harris *et al.*, 1992). However, nisin's poor solubility above pH 5 and instability in some foods limits its use to foods such as processed cheeses, dairy products, and canned foods (Delves-Broughton, 1990) and opens the field to discovery of other broad spectrum bacteriocins that can overcome these hurdles. The ability of nisin to control the growth of spoilage and pathogenic organisms on fresh and cooked meats is limited (Chung *et al.*, 1989; El-Khateib *et al.*, 1993).

2.2.2.2 Nonlantibiotics

Most recently identified bacteriocins belong to the class II group of nonlantibiotic heat stable peptides. These include lactacin F from *Lactobacillus johnsonii*, pediocin PA-1/AcH from *Pediococcus acidilactici*, curvacin A from *Lactobacillus curvatus*, sakacin A from *Lactobacillus sake*, sakacin P from *Lactobacillus sake*, leucocin A-UAL 187 from *Leuconostoc gelidum*, and lactococcins A,B, and M from *Lactococcus lactis*. Heat resistance of class II bacteriocins can vary from 100°C for more than 30 min to autoclaving at 121°C for 15 to 20 min. These peptides have been purified and their structural genes (genes encoding the respective bacteriocin peptide) have been identified. Genetic analysis has shown that these peptides are synthesized as prebacteriocins with an N-terminal extension of 18 to 24 residues (see results p. 74) that is cleaved by a leader peptidase after a characteristic Gly-Gly site to release the mature bacteriocin. Subgroups that can be defined within the class II bacteriocins are: (a) *Listeria* - active peptides with a consensus sequence of -TYR-GLY-ASN-GLY-VAL- ("YGNGV"

motif) in the N-terminal region of the mature peptide (Hastings *et al.*, 1991; Holck *et al.*, 1992; Lozano *et al.*, 1992; Tichaczek *et al.*, 1992), (b) peptides that require the complementary activity of two peptides for biological activity (Nissen-Meyer *et al.*, 1992; Allison *et al.*, 1993; van Belkum *et al.*, 1991), and (c) peptides that require reduced cysteine residues for activity (Venema *et al.*, 1993).

2.2.3 The potential for LAB bacteriocins to be used as natural preservatives.

LAB bacteriocins show promise for use as a natural means of food preservation. Although their inhibitory spectra are limited thus far to Gram-positive bacteria, several bacteriocins produced by LAB have been shown to be active against food spoilage and foodborne pathogenic microorganisms, including *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens*, *Listeria monocytogenes*, and *Staphylococcus aureus*. Class II bacteriocins have been used successfully as antimicrobials in some meat applications. These include pediocins to control the growth of *Listeria monocytogenes* in both fresh meats (Motlagh *et al.*, 1992; Nielsen *et al.*, 1990) and processed meats (Berry *et al.*, 1991; Degnan and Luchansky, 1992), and sakacin A to inhibit growth of *L. monocytogenes* on pasteurized ground meat and fresh sausage (Schillinger *et al.*, 1991). In addition, some bacteriocins (e.g. nisin) have been shown to inhibit Gram-negative species that have been presensitized with chelating agents such as EDTA (Kalchayanand *et al.*, 1992) which is thought to alter the permeability of the cell wall.

Many bacteriocins are heat stable so that biological activity is maintained

after heat treatment. This may allow their use in heat processed foods, with a concomitant reduction in intensity of the thermal process and improvements in the nutritional and organoleptic properties of the food. Finally, bacteriocins are produced by LAB that have been used to ferment foods for many years and suggests the nontoxic nature of bacteriocins produced by these organisms. Indigenous bacteriocin-producing LAB are commonly found in retail foods and suggests that the public is already consuming viable LAB on ready-to-eat products (Garver and Muriana, 1993). Bacteriocins are thought to confer a competitive advantage to a producing organism against others within the same ecological niche (Ahn and Stiles, 1990).

2.2.4 Genetic components necessary for bacteriocin production.

To achieve the goal of using natural antimicrobials to contribute to the safety of foods by antagonism of foodborne pathogens, the bacteriocins discovered need to be fully characterized both biochemically to assess their potential for use in a given food system, and genetically to ensure that the bacteriocins are different from those already discovered. Problems that might arise from the use of purified bacteriocins in a food system such as meat include undesirable sensory changes and (or) inactivation of the bacteriocin by proteolytic enzymes present within the meat environment. Newly discovered bacteriocins also need to have their amino acid sequence determined because identical bacteriocins may be produced by related organisms that differ in their source of isolation. This was the case for the

bacteriocins pediocin PA-1 and pediocin AcH, in which the *Pediococcus acidilactici* strains were isolated from different sources but produced the identical bacteriocin. This implied a common industrial origin for the organisms.

For each bacteriocin, the structural gene can be localized to either the genomic or plasmid DNA if present. Revealing the structural gene allows: (1) deduction of the (complete) amino acid structure of the peptide and confirmation of the proteinaceous nature of the purified compound; (2) deduction of the N-terminal extension of the prebacteriocin; (3) prediction of the secondary structures that may form; and (4) postulation of the mode of action through homology with other bacteriocins. Other genes that aid in immunity to and in the secretion of the bacteriocin may also be revealed because these are usually located in close proximity to the structural gene in an operon-like organization. In addition, by determining the genetic information of bacteriocins with different antibacterial spectra, biochemical properties, and modes of action, it is theoretically possible to use recombinant DNA technology to develop novel methods for enhanced control of spoilage and pathogenic bacteria. To be of practical significance, the recipient of the genetic information should be a food-grade organism with low spoilage potential, nonpathogenic, and is able to demonstrate predictable growth and bacteriocin production in the chosen food system.

2.3 POTENTIAL APPLICATION FOR BROCHOCIN-C TO CONTROL SPOILAGE AND PATHOGENIC MICROORGANISMS.

2.3.1 Introduction

Prior to 1988, *B. thermosphacta* was the only species in the genus *Brochothrix*. Through taxonomic and DNA hybridization studies of a number of strains of *Brochothrix* spp. isolated from a variety of sources, including soil and grass, Talon *et al.* (1988) identified and described a second species, *B. campestris*. Apart from its initial discovery and characterization, limited information on *B. campestris* is available. To date, *B. campestris* has only been isolated from soil and grass. However, it is possible that in the past the species was misidentified as *B. thermosphacta*, owing to the fact that *B. thermosphacta* has also been isolated from the same habitats. Differentiating features of *B. thermosphacta* from *B. campestris* are that the latter species is unable to grow in 8% NaCl in 2 d, it is able to hydrolyze hippurate, and it is able to produce acid from rhamnose (Jones, 1992). While the habitat of *Brochothrix* spp. is not known with certainty, it is probable that they are widely distributed in the environment and that they become a prominent part of the microflora in habitats that selectively favour their growth.

2.3.2 Brochocin-C production by *B. campestris* ATCC 43754.

Siragusa and Nettles Cutter (1993) reported an inhibitory substance produced by *B. campestris* ATCC 43754. This substance was reported to be proteinaceous in nature, was identified as a bacteriocin, and named brochocin-C. In their study, brochocin-C was found to be active against isolates from vacuum

packaged beef and pork. Using cell free supernatants of a 16 h culture grown in tryptic soy broth plus 0.5% (w/v) yeast extract, they found that all of the 35 strains of *B. thermosphacta* and 10 strains of *Listeria monocytogenes* were inhibited by brochocin-C. Inhibitory activity was also reported against strains of *Carnobacterium*, *Enterococcus*, *Kurthia*, *Lactobacillus*, and *Pediococcus*. No activity was detected against the Gram-negative species. The significance of bacteriocin production by *B. campestris* in nature has yet to be determined, but it is likely that it offers the organism some competitive advantage in its ecological niche. It is interesting to note that the inhibitory spectrum of brochocin-C includes organisms commonly found in meat and other foods, yet the producing organism has not been documented as part of the meat microflora.

The fact that bacteriocin production has been associated with a member of the genus *Brochothrix* expands the range of Gram-positive bacteria that produce bacteriocins. Many of the bacteriocins of LAB characterized to date have a narrow spectrum of activity that is usually restricted to closely related organisms. The relatively broad spectrum of activity of brochocin-C warrants further investigation of the nature of its biological activity.

3. Materials and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in these studies are listed in Table 1. These include strains from the American Type Culture Collection (ATCC), *Brochothrix* strains from G.G. Greer isolated from meat at the Lacombe Research Centre and from our laboratory culture collection (UAL). All strains with the exception of *Escherichia coli* were stored at -70°C in All Purpose Tween (APT) broth (Difco Laboratories Inc., Michigan) adjusted to pH 6.5, supplemented with 20% glycerol (v/v). Cultures for use in experimental studies were obtained by inoculation of frozen cells into APT broth at pH 6.5, and subcultured for two successive transfers at 25°C after 18 to 24 h before being used. Growth experiments and (or) bacteriocin production from *B. campestris* were done in APT broth, Cooked Meat Medium (CMM; Difco), or semi-defined casamino acids medium (CAA), described by Hastings *et al.* (1991). CAA medium was used for the purification of the bacteriocin.

E. coli strains were stored at -70°C in Luria-Bertani (LB) broth (Sambrook *et al.* 1989) supplemented with 40% glycerol (v/v). Inoculation of *E. coli* strains was done from frozen cultures into LB broth with ampicillin or erythromycin added to a final concentration of 200 μg/mL and propagated at 37°C with shaking (250 rpm). Potential pUC118 recombinants were identified by the blue-white colour selection from growth on LB plates (1.5% w/v granulated agar) supplemented with ampicillin (200 μg/mL) and used with X-gal (5-bromo-4-chloro-

Table 1. Bacterial strains and plasmids used in this study

Organism	Reference
Bacillus macerans ATCC 7048	ATCC
B. cereus ATCC 14579	ATCC
Brochothrix campestris ATCC 43754	ATCC
B. campestris MT	This study
B. thermosphacta ATCC 11509	ATCC
B. thermosphacta I41	UAL
B. thermosphacta B1 - B5, B7 - B16 (inclusive)	GGG
Carnobacterium piscicola LV17	Shaw
C. piscicola LV17A	Ahn and Stiles (1990)
C. piscicola LV17B	Ahn and Stiles (1990)
C. piscicola LV17C	Ahn and Stiles (1990)
C. piscicola C2/8B	Quadri *et al.* (1994)
C. piscicola C2/8A	Quadri *et al.* (1994)
C. piscicola UAL26	Burns (1987)
C. piscicola UAL26/8A	Ahn and Stiles (1990)
C. piscicola UAL26/8B	Quadri *et al.* (1994)
C. divergens LV13	Shaw
C. divergens 9/8A	Quadri *et al.* (1994)
C. divergens 9/8B	Quadri *et al.* (1994)
Clostridium bifermentans ATCC 19299	ATCC
C. butyricum ATCC 8260	ATCC
C. pasteurianum ATCC 6013	ATCC
Enterococcus faecalis ATCC 19433	ATCC
E. faecalis ATCC 7080	ATCC
E. faecium ATCC 19434	ATCC
E. durans ATCC 11576	ATCC
Lactobacillus sake Lb706	Schillinger
L. plantarum ATCC 4008	ATCC
Lactococcus lactis ATCC 11454	ATCC
L. lactis UAL 245	UAL
L. lactis UAL 276	UAL
Leuconostoc gelidum UAL 187	Hastings *et al.* (1991)
L. gelidum UAL 187.13	Hastings *et al.* (1991)

L. gelidum UAL 187.22		Hastings *et al.* (1991)
L. mesenteroides ATCC 23386		ATCC
L. mesenteroides Y105		Cenatiempo
Listeria innocua ATCC 33090		ATCC
L. monocytogenes Scott A		ATCC
L. monocytogenes I42		UAL
L. monocytogenes ATCC 15313		ATCC
Pediococcus acidilactici ATCC 8042		ATCC
P. acidilactici PAC 1.0		Vandenbergh
Staphylococcus aureus S6		HPB
S. aureus S13		HPB
Escherichia coli DH5-α		BRL Laboratories Life Technologies Inc.
E. coli	AP4.7 (DH5-α containing pAP4.7)	This study
E. coli	AP7.4-32 (DH5-α containing pAP7.4)	This study
E. coli	AP4.6-8 (DH5-α containing pAP4.6)	This study
Plasmids		
pUC118	(3.2kb; Amp^R; lac Z/)	Vieira and Messing, (1982)
pGKV210	(4.4kb; Em^R)	van der Vossen *et al.* (1985)
pAP4.7	(pUC118; 1.6 kb *Eco*RI - *Pst*I fragment)	This study
pAP7.4	(pUC118; 4.2kb *Eco*RI fragment)	This study
pAP4.6	(pUC118; 1.4kb *Pst*I fragment)	This study
pAP8.6	(pGKV210; 4.2 kb *Eco*RI fragment)	This study

ATCC = American Type Culture Collection
BRL = Bethesda Research Laboratories Life Technologies Inc.
UAL = University of Alberta Food Microbiology culture collection
GGG = G. Gordon Greer (Lacombe Research Centre, Alberta, Canada)
HPB = Health Protection Branch (Ottawa, Ontario, Canada)
Shaw = B. G. Shaw (AFRC Institute of Food Research, Bristol, UK)
Vandenbergh = P. A. Vandenbergh (Quest International, Sarasota, US)
Burns = K. Burns (M.Sc. thesis, 1987, University of Alberta, Edmonton, AB)
Schillinger = U. Schillinger (Institute of Hygiene and Toxicology, Federal Research Centre for Nutrition, Karlsruhe, Germany)
Cenatiempo = Y. Cenatiempo (Institut de Biologie Moléculaire et d'Ingénierie Génétique, Centre National de la Recherche Scientifique, Université de Poitiers, France)

3-indolyl-ß-D-galactopyranoside) and IPTG (isopropylthio-ß-D-galactopyranoside) at final concentrations of each at 1.6 mg/mL. Erythromycin-resistant (Em^R) transformants of *E. coli* with pGKV210 were selected on either LB or YT (yeast extract, tryptone; Difco) agar with erythromycin (200 μg/mL).

Bacteriocin assays

Antagonistic bacteriocin activity against different indicator strains was determined by direct or deferred inhibition assays (Ahn and Stiles, 1990). For direct inhibition tests, broth cultures were inoculated onto APT agar (1.5%) plates using a Cathra replicator, allowed to dry, and immediately overlayered with 7.5 mL of molten APT agar (0.75% agar) at 45°C, seeded with a 1% inoculum of the indicator strain. For deferred inhibition tests, inoculated cells were incubated at 25°C for 15 to 18 h before being overlayered with the indicator strain as described above. In both instances, overlayered plates were placed in an anaerobic jar (BBL) filled with a 10% CO_2 and 90% N_2 atmosphere and incubated at 25°C for 16 to 24 h before analyzing the results.

Bacteriocin activity of *B. campestris* ATCC 43754 was detected or quantified by the spot-on-lawn method (Ahn and Stiles, 1990) against *C. piscicola* LV17C. Doubling dilutions (1:1) of cell supernatants (heat treated at 65°C for 30 min) were prepared in sterile water and 10 or 20 μL of each dilution was spotted onto an APT plate freshly overlayered with the indicator lawn. Activity was determined by taking the reciprocal of the highest dilution which showed a distinct zone of

inhibition of the indicator strain, and expressed as arbitrary activity units (AU) per mL.

Stability of brochocin-C

The effects of pH and heat treatment on the activity of crude brochocin-C were determined. Cultures grown in APT broth were centrifuged (8000 x g for 15 min) and the supernatant was adjusted to pH 2 through 9 using either 5 N HCl or NaOH. The pH-adjusted supernatant was heated at 65°C for 30 minutes before doing a spot-on-lawn assay. Heat stability of brochocin-C in pH-adjusted supernatant was determined by heating at 65°C for 30 min, 100°C for 15 min, or 121°C for 15 min before testing the residual activity of each sample and comparing it with the activity in unheated supernatant. To test the effect of organic solvents on the activity of brochocin-C, preparations of brochocin-C partially purified by butanol extraction (see below) were diluted in either 0.1% trifluoroacetic acid (TFA), 95% ethanol, 100% methanol, or 100% acetonitrile to give an initial concentration of 10 AU/μL. Tubes were incubated at 25 and 4°C for selected time intervals before a 10 μL aliquot of each treatment was removed and spotted onto a freshly overlayered lawn of *C. piscicola* LV17C. Sizes of the zones of inhibition were measured and compared to that at time zero for each treatment.

Plasmid curing

Overnight cultures of *B. campestris* were inoculated at 10^7 cfu/mL into APT

broth containing different concentrations of the curing agents novobiocin, acriflavin, and sodium-dodecyl sulphate (SDS) and grown at 25°C for 24 h to determine the minimum inhibitory concentration of each.

The loss of bacteriocin production was determined from cultures grown in acriflavin by heating a 500 μL aliquot of the culture at 65°C for 30 min and spotting it onto a lawn of *C. piscicola* LV17C. A negative control of sterile APT broth with the different concentrations of acriflavin was also spotted onto the indicator lawn to ensure that the acriflavin did not have an inhibitory effect on the indicator cells. Curing was attempted using a combination of acriflavin and elevated growth temperature (30°C) using an inoculum of 10^4 cfu/mL in APT broth with the selected acriflavin concentration. The culture was grown until turbidity was detected and then it was subcultured an additional 1 to 6 times at inocula of 10^3 or 10^4 cfu/mL in APT broth containing the same acriflavin concentrations. Dilutions of these cultures were made in sterile 0.1% peptone (Difco) water and plated onto APT plates. Plates were incubated in anaerobic jars at 25°C for 2 d and replica-plated onto two other APT plates, allowed to grow for 2 d before overlayering one plate with *C. piscicola* LV17C and the other with *Listeria monocytogenes* 33090. Colonies showing a loss of bacteriocin production with both of the indicator strains were inoculated into APT broth for small-scale plasmid isolation (see below). The wild-type strain was also included in the small-scale plasmid isolations to serve as a positive control.

Purification of brochocin-C

A flask containing five litres of sterile CAA medium (Hastings *et al.*, 1991) with 2.5% glucose was inoculated with 2% of an overnight culture of *B. campestris* ATCC 43754, and grown at a constant pH of 6.7 with a Chemcadet (Cole-Parmer, Chicago, IL) by addition of filter-sterilized (0.22μm) 2 M NaOH. Growth of the culture was monitored and stopped after 22 h of incubation at 25°C. Cells were removed from the culture broth by centrifugation at 8000 x g for 20 min. The supernatant (approximately 5.5 litres) was extracted twice with 1.5 litres of n-butanol. The extract was diluted with water (approximately 1:1), concentrated on a vacuum evaporator at 35°C and evaporated repeatedly to remove the last traces of butanol. The extract was suspended in water (approximately 150 mL), precipitated with 1.7 litres of cold (-60°C) acetone and stored at 5°C for 24 h. The precipitate was separated by centrifugation (10,000 x g for 15 min), dissolved in 10 mL of 0.1% TFA and loaded onto a Sephadex G50 (Pharmacia) column (2.5 x 120 cm) that had been pre-equilibrated with 0.1% TFA. The column was washed with 0.1% TFA at a flow rate of approximately 0.6 mL/min. Absorbance of collected fractions was monitored at 220 nm. Fractions showing antimicrobial activity by spot-on-lawn assay were concentrated and lyophilized. The purity of the sample was confirmed by mass spectrum analysis and sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Brochocin-C preparations were examined on 20% polyacrylamide gels with the buffer system described by Laemmli (1970) in 3M Tris-HCl. Electrophoresis was done at 20 mA constant current for 3 h. After electrophoresis, gels were fixed in 50% methanol, 10% acetic acid for 1 h and stained with Coomassie blue (Bio-Rad) or assayed for antimicrobial activity by overlayering with *C. piscicola* LV17C as the indicator strain by the method of Barefoot and Klaenhammer (1983).

Inhibition by brochocin-C

Partially purified preparations of brochocin-C were obtained by butanol extraction of supernatant fluids of an overnight culture of *B. campestris* ATCC 43754 grown in CAA medium with constant pH regulation at 6.7. All traces of butanol were removed by rotary evaporation. The partially purified bacteriocin was added to APT broth (pH 6.5) and to phosphate buffer (50 mM, pH 7.0) containing 10^6 cfu per mL of *C. piscicola* LV17C. The bacteriocin was added to give a final concentration of 100 AU/mL and the tubes were incubated at 25°C. Viable counts were determined by enumeration on APT agar at selected time intervals and cell lysis was checked by monitoring the optical density at 600 nm. For enumeration, cultures grown in APT broth and phosphate buffer were diluted in sterile 0.1% peptone water and 50 mM phosphate buffer (pH 7.0) respectively. Growth of the indicator strain without addition of bacteriocin was also included as a control.

Determination of the amino acid sequence and the amino acid content of brochocin-C

The N-terminal amino acid sequence of brochocin-C was determined by the Alberta Peptide Institute (API; University of Alberta, Edmonton, AB) by automated Edman degradation with a gas-phase sequencer (Applied Biosystems model 470A) with on-line phenylthiohydantoin-derivative identification by HPLC (Applied Biosystems model 120A chromatograph). They also determined the amino acid content of purified brochocin-C by derivitization with phenylisothiocyanate on an Applied Biosystems 420A derivatizer and separation with a C_{18} column by HPLC (Applied Biosystems model 130A chromatograph).

The mass spectrum of purified brochocin-C was measured by plasma desorption and fast atom bombardment (FAB).

DNA isolation, manipulation, and hybridization

Small-scale plasmid isolation of *B. campestris* was done by previously established methods (Ahn and Stiles, 1990). Cells from an overnight culture grown in APT broth were recovered by centrifugation at 14,000 rpm for 5 min, washed once with cold 0.5% NaCl (500 μL), and resuspended in 100 μL of solution A (25% sucrose, 50 mM Tris-HCl, 5 mM EDTA, pH 8.0) containing lysozyme (10 mg/mL). After incubation for 1 h at 37°C, 200 μL of solution C (0.9% glucose, 3% SDS, 50 mM Tris-HCl, 5 mM EDTA, pH 8.0) containing 0.2 N NaOH was added and the tubes were gently inverted several times until the cell lysate cleared. Solutions of 2 M Tris-HCl, pH 7.0 (50 μL) and 5 M NaCl (70 μL) were added to the tubes

and mixed by inversion. The DNA was extracted once with 3% NaCl-saturated phenol/chloroform and once with chloroform/isoamyl-alcohol (24:1), before overnight precipitation at -20°C with 95% ethanol. Large-scale preparation of plasmid DNA was done by scaling up (100 X) of the small-scale method using cells from 750 mL of an overnight culture grown in APT broth and purified by CsCl - ethidium bromide density gradient ultracentrifugation. The CsCl salt was removed by dialysis in 10 mM Tris-HCl, 1 mM EDTA (TE buffer, pH 8.0; Sambrook *et al.*, 1989). Chromosomal DNA preparation of *B. campestris* was done as described by Quadri *et al.* (1994), but was resuspended in a final volume of 1 mL TE buffer. An equal volume of chloroform was added to preserve the DNA from bacterial contamination and to remove any residual proteins.

Plasmid and genomic DNA from *B. campestris* was digested with restriction enzymes compatible with the multiple cloning site (MCS) of pUC118 (Vieira and Messing, 1982). Restriction endonucleases from Boehringer-Mannheim (Dorval, Quebec, Canada), Promega (Madison, WI; Burlington, Ontario, Canada), and New England Biolabs (Mississauga, Ontario, Canada) were used as recommended by the suppliers. DNA fragments were separated in either 0.65% 40 mM Tris-acetate/1 mM EDTA (TAE) or 0.7% 90 mM Tris-borate/2 mM EDTA (TBE) agarose gels run at 8.5V/cm and blotted by the method of Southern (1975) onto Hybond N (Amersham Corp.) nylon membranes. Molecular weights of fragments were determined by multiple regression analysis based on mobility standards of *Eco*RI - *Hin*dIII digests of bacteriophage lambda (Promega).

For colony blots, Hybond N membrane was placed on top of the colonies, lifted off the plate, incubated for 6 to 8 h on a new LB-ampicillin plate, where necessary, to allow growth of the cells, and the colonies were lysed on the membrane *in situ*.

A degenerate 23-mer oligonucleotide probe, (APO-1; 5'-AAAGATATTGG(ATC)AAAGG(ATC)ATTGG -3') based on residues 8 to 15 of the amino acid sequence, was used to locate the brochocin-C structural gene (*brcC*) in both Southern and colony blot hybridizations. Oligonucleotides based upon derived nucleotide sequences were synthesized as needed (Department of Biological Sciences, University of Alberta, Edmonton, AB) on an Applied Biosystems 391 PCR Mate synthesizer, quantified, and used for hybridizations or as primers for nucleotide sequencing without further purification. DNA probes were radioactively end-labelled with [gamma-^{32}P]ATP (Amersham) with T4 polynucleotide kinase (PNK; Promega) or nonradioactively by random-primed labelling with digoxigenin-dUTP (Boehringer-Mannheim). A reaction volume of 10 μL of the labelled oligonucleotide mixture (6 μL distilled water, 1 μL 10X PNK buffer, 1 μL [1 pmol] APO-1 probe, 1 μL PNK, 1 μL [gamma-^{32}P] ATP) was added for every 3 mL of hybridization solution. The mixture was purified through a Sephadex G50 column to remove unincorporated ATP or added directly to the hybridization solution. Hybridizations were done at 37°C overnight in hybridization solution containing 6X SSPE buffer, 5X Denhardt's Reagent (Sambrook *et al.*, 1989) and 0.5% (v/v) SDS. After hybridization, two washes were

done sequentially (25°C for 25 min, 39°C for 15 min) in 2X SSPE buffer, 0.1% SDS. Where necessary, probes were stripped off membranes by washing at 95°C for 2 min in 0.5% SDS and rehybridized. Autoradiograms were exposed 24 to 48 h before developing in a Fuji film processor.

Isolation of small-scale plasmid DNA from *E. coli* strains was performed by the lysis by boiling method and large-scale DNA preparation by alkaline lysis (Sambrook *et al.*, 1989). Large-scale plasmid DNA was purified by equilibrium centrifugation at 49 000 rpm (Ti 70.1 rotor) for 20 h in a CsCl-ethidium bromide gradient and dialyzed in TE buffer.

Cloning of the *brcC* gene

Genomic DNA was digested to completion with *Eco*RI. Fragments of 4.2 kb corresponding to the hybridization signal identified with APO-1 were excised from the gel and placed in 6,000 to 8,000 molecular weight cut-off Spectrapor (Los Angeles, CA) dialysis tubing. The DNA was electroeluted from the gel and into the tubing by electrophoresis at 200V for 20 min in 0.5% TBE buffer. The DNA was purified by extracting once with phenol/chloroform:isoamyl alcohol (24:1), once with chloroform:isoamyl alcohol, and precipitated with 2 volumes of 95% ethanol and one-tenth volume of 3 M sodium acetate (pH 5.2). The resulting fragments were cloned into the *Eco*RI site of the MCS in pUC118 using T4 DNA ligase (Promega) at 25°C for 3 h in ligation buffer without polyethylene glycol and dithiothreitol. Colonies were screened by α-complementation (Vieira and Messing, 1982). Colony blots were done to discriminate the white colonies for the correct

DNA insert. Small-scale plasmid isolations were done on presumptive positive clones and the plasmids were digested with *Taq*I. The clones were grouped into classes based on similarities in their restriction patterns. Clones were digested with *Eco*RI, blotted by the method of Southern (1975), and hybridized with APO-1 to confirm the presence of the *brcC* gene. The plasmid identified to carry the correct 4.2 kb insert in pUC118 was named pAP7.4. A smaller *Pst*I fragment of 1.4 kb was further identified from this plasmid to hybridize to APO-1 and this was subcloned into pUC118 (pAP4.6).

Nucleotide sequencing of plasmid DNA

The plasmid pAP4.6 served as the initial template DNA for nucleotide sequencing by *Taq* DyeDeoxy Cycle sequencing (Department of Biochemistry, University of Alberta, Edmonton, AB) on an Applied Biosystems 373A sequencer using the universal forward and reverse primers of pUC118. Site-specific 18-mer primers based on newly sequenced DNA were synthesized for further sequencing. The recombinant plasmid, pAP7.4, was used as the template DNA in subsequent sequencing runs to deduce the complete sequence of the structural gene (*brcC*), the regions flanking the structural gene, and for sequencing of the complementary strand.

Heterologous and homologous expression studies of brochocin-C

The 4.2 kb insert in pAP7.4 was subcloned into the *Eco*RI site of the shuttle

vector pGKV210 to create the recombinant plasmid pAP8.6. This plasmid was subsequently used to transform selected strains by electroporation with a Gene-Pulser (Bio-Rad Laboratories Canada Ltd., Mississauga, ON) at 25 μFD and 200 ohms resistance.

4. Results

4.1 PHYSIOLOGICAL CHARACTERIZATION OF *B. campestris* ATCC 43754.

Inhibitory spectrum of *B. campestris*

The inhibitory spectrum of brochocin-C was determined against various indicator strains by direct and deferred antagonism tests (Table 2). *Brochothrix campestris* ATCC 43754 was active against all strains of *Brochothrix*, *Carnobacterium*, *Enterococcus*, and *Listeria* tested but was immune to its own bacteriocin. No inhibition was observed against the *Pseudomonas aeruginosa* strain tested. Inhibitory activity was also not observed against strains of *Aeromonas hydrophila* ATCC 7466, *Salmonella typhimurium* ATCC 4028, *Shewanella putrefaciens* ATCC 8071, *E. coli* ATCC 25922, and *Pseudomonas fragi* JU14 (Leisner, unpublished data). The bacteriocin was active against spores of *Clostridium botulinum* (McMullen and Stiles, unpublished data).

Growth and production of brochocin-C.

Brochothrix campestris ATCC 43754 grown in APT broth at 25°C with an initial inoculum of approximately 10^7 cfu/mL and a starting pH of 6.5 resulted in detectable bacteriocin production after 3 h (Fig. 2). While maximum population density was achieved after 18 h of growth, maximum bacteriocin production was not detected until late in the stationary phase of growth (30 h) but retained the same level of activity for up to 48 h. The pH, after 48 h of incubation of *B.*

Table 2. Inhibitory spectrum of Brochothrix campestris ATCC 43754 determined by direct and deferred antagonism on APT agar

Indicator	Direct*	Deferred*
Bacillus macerans ATCC 7048	—	—
B. cereus ATCC 14579	++	+++
Brochothrix campestris ATCC 43754	—	—
B. thermosphacta B1	++	++++
B. thermosphacta B2	++	++++
B. thermosphacta B3	++	++++
B. thermosphacta B4	++	++++
B. thermosphacta B5	++	++++
B. thermosphacta B7	++	++++
B. thermosphacta B8	++	++++
B. thermosphacta B9	++	++++
B. thermosphacta B10	++	++++
B. thermosphacta B11	++	++++
B. thermosphacta B12	+	++++
B. thermosphacta B13	++	++++
B. thermosphacta B14	+	++++
B. thermosphacta B15	++	++++
B. thermosphacta B16	+	++++
B. thermosphacta L90	+	++++
B. thermosphacta NF4	++	++++
B. thermosphacta C420	+	++++
B. thermosphacta 141	++	+++
Carnobacterium piscicola LV17	++++	++++
C. piscicola LV17A	++++	++++
C. piscicola LV17B	++++	++++
C. piscicola LV17C	++++	++++
C. piscicola C2/8B	++++	++++
C. piscicola C2/8A	++++	++++
C. piscicola UAL26	+++	++++
C. piscicola UAL26/8A	+++	++++
C. piscicola UAL26/8B	++++	++++
C. divergens LV13	+++	++++
C. divergens 9/8A	+++	++++
C. divergens 9/8B	+++	++++

Clostridium bifermentans ATCC 19299	+++	++++
C. butyricum ATCC 8260	ND	+++
C. pasteurianum ATCC 6013	ND	+++
Enterococcus faecalis ATCC 19433	+++	++++
E. faecalis ATCC 7080	+++	+++
E. faecium ATCC 19434	+++	++++
E. durans ATCC 11576	+++	++++
Lactobacillus sake Lb706	+++	++++
L. plantarum ATCC 4008	—	—
Lactococcus lactis ATCC 11454	—	+
L. lactis UAL 245	+	+
L. lactis UAL 276	ND	+
Leuconostoc gelidum UAL 187	++	+++
L. gelidum UAL 187.13	+	++
L. gelidum UAL 187.22	++	+++
L. mesenteroides ATCC 23386	—	—
L. mesenteroides Y105	—	++
Listeria innocua ATCC 33090	++	+++
L. monocytogenes Scott A	+++	++++
L. monocytogenes UAL 42	++	+++
L. monocytogenes ATCC 15313	+	++
Pediococcus acidilactici ATCC 8042	+	++
Staphylococcus aureus S6	++	++
S. aureus S13	++++	++++

* ++++ = zone of inhibition ≥20 mm
+++ = zone of inhibition 15 to 19 mm
++ = zone of inhibition 10 to 14 mm
\+ = zone of inhibition 5 to 9 mm
– = no inhibition zone

ND = not determined

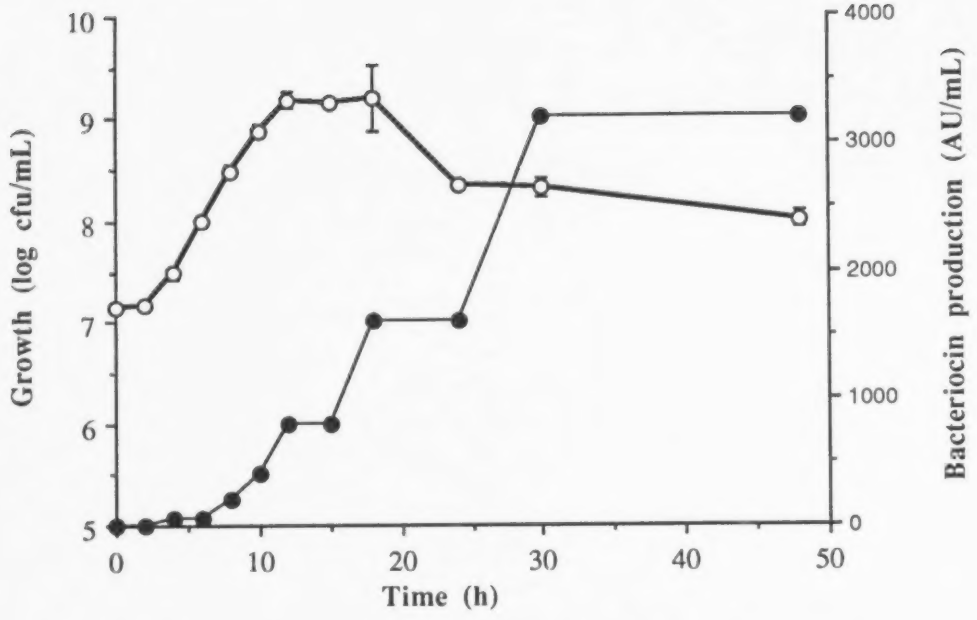

Figure 2. Growth and bacteriocin production by *Brochothrix campestris* ATCC 43754 over time in APT broth (pH 6.5) at 25°C.

Growth (o) is shown in $\log_{10}$ cfu/mL and bacteriocin titres (●) were determined against *C. piscicola* LV17C. Error bars represent standard deviation for two data points.

campestris ATCC 43754, was 5.0.

Growth in the semidefined casamino acids (CAA) medium used by Hastings *et al.* (1991) without pH adjustment (pH 6.5) resulted in a four-fold decrease in the maximum level of bacteriocin production (Fig. 3a) compared with production in APT broth, although the maximum population reached is only a 0.5 log unit lower in CAA. The final pH in APT broth after 48 h and in CAA after 51 h was identical. When CAA medium was adjusted to pH 7.0 just prior to inoculation and maintained throughout the growth period with 2 N NaOH, a five-fold increase in the level of bacteriocin production, accompanied by a one log increase in the maximum cell density was observed as compared with CAA medium without regulation of pH (Fig. 3b).

Growth in cooked meat medium (CMM; pH 6.5) at 25°C showed poor growth, achieving only a one log increase in cell density when inoculated at approximately 10^7 cfu/mL and no detectable amounts of bacteriocin in the supernatant fluid even after 36 h growth (Fig. 4). Growth was rapid with the inoculum at 10^4 cfu/mL but only reached a level of 10^7 cfu/mL with again, no detectable levels of bacteriocin activity.

These results suggest that pH plays an important role in production of the bacteriocin and that a critical cell density of approximately 10^8 cfu/mL is necessary for brochocin-C to be produced or before it can be detected by the limits of our assay. However, it is possible that if the bacteriocin is produced, it may be inactivated by some mechanism such as through binding to the meat particles.

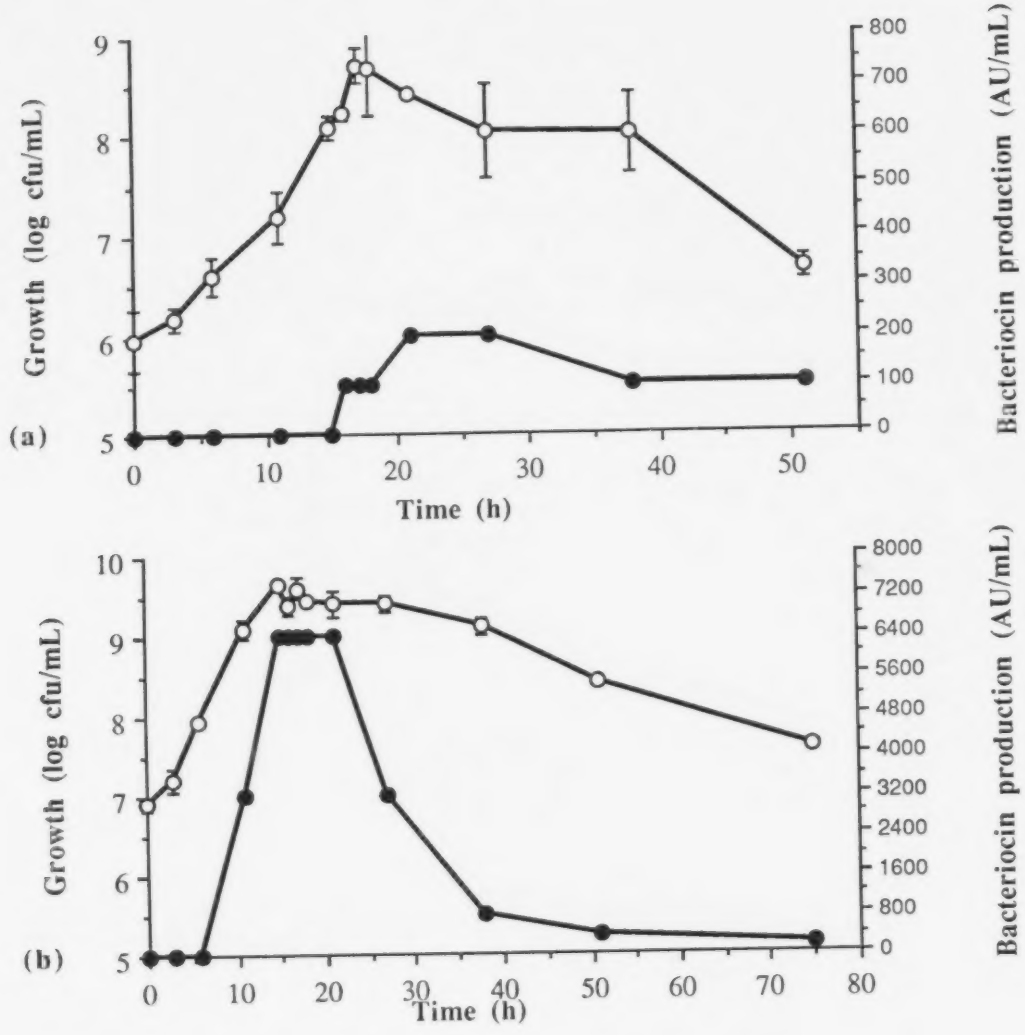

Figure 3. Growth and bacteriocin production by *Brochothrix campestris* ATCC 43754 in CAA medium over time at 25°C with: (a) no regulation of pH and (b) pH regulated at 7.0 with 2 N NaOH.

Growth (o) is reported in $\log_{10}$ cfu/mL and bacteriocin titres (●) were determined against *C. piscicola* LV17C. Error bars represent standard deviation for two data points.

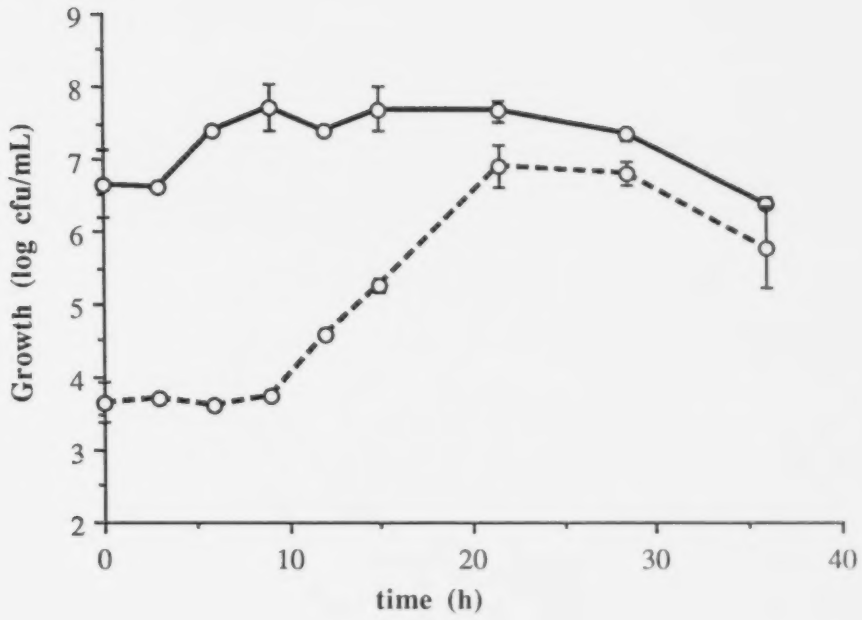

Figure 4. Growth of *Brochothrix campestris* ATCC 43754 in CMM incubated at 25°C with a starting inoculum of 10^7 cfu/mL (-o-) and 10^4 cfu/mL (--o--). Error bars represent standard deviation for two data points.

4.2 BIOCHEMICAL CHARACTERIZATION OF BROCHOCIN-C

pH and thermal stability of brochocin-C.

The levels of bacteriocin present in the supernatant fluids of an 18 h culture grown in APT adjusted to initial pH levels between 4.5 and 7.0 are shown in Fig. 5. Similar levels of inhibitory activity were observed against all three of the indicator strains with the greatest bacteriocin production occurring in APT broth adjusted to pH ≥6.0. A 36 h culture of *B. campestris* showed little if any increase in the level of bacteriocin production against the same indicator strains.

The bacteriocin present in the supernatant fluids of an 18 h culture grown in APT broth at 25°C was thermal stable from pH 2 to 9 (Fig. 6). This is significant in comparison to other bacteriocins such as nisin which has poor solubility above pH 5.0 and thus inactive. The same level of bacteriocin activity for each pH value was observed whether the sample was unheated, heated at 65°C for 30 min, or boiled at 100°C for 15 min. Even after a 1 week storage period at 4°C, the samples heated at 100°C for 15 min still had the same levels of bacteriocin activity. A decrease in the bacteriocin activity was only seen when the pH-adjusted supernatant fluid was heated at 121°C for 15 min, with the effect being most pronounced at pH 9.0.

Inhibition by brochocin-C.

Partially purified bacteriocin was added at a concentration of 100 AU/mL to APT broth (pH 6.5) and phosphate buffer (50 mM, pH 7.0) containing *C. piscicola*

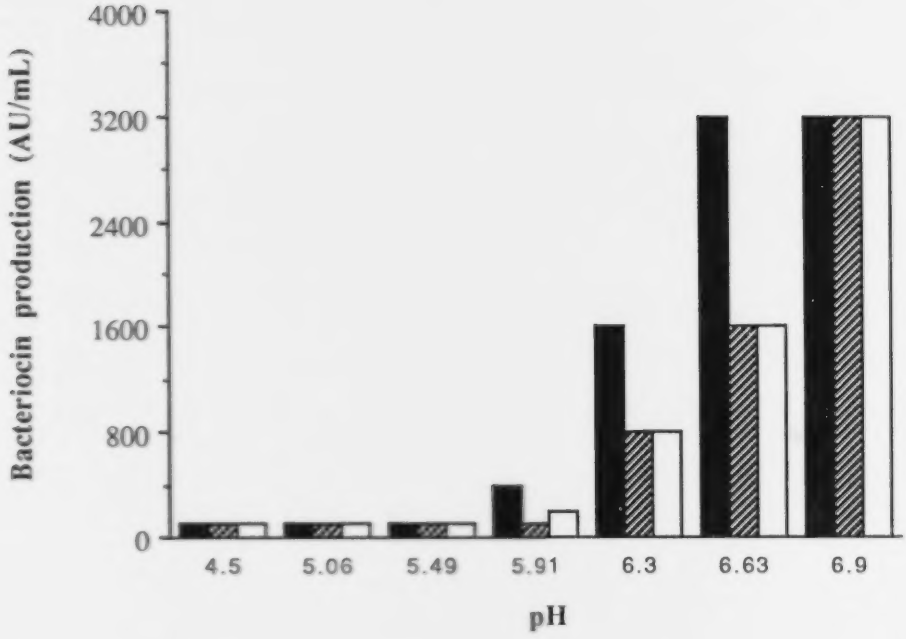

Figure 5. The effect of initial pH of APT broth on bacteriocin production by *Brochothrix campestris* ATCC 43754 incubated at 25°C.

Supernatant fluids from an 18 h culture were tested against *Carnobacterium piscicola* LV17C (■), *Carnobacterium divergens* LV13 (▨), and *Enterococcus faecalis* ATCC 19433 (□).

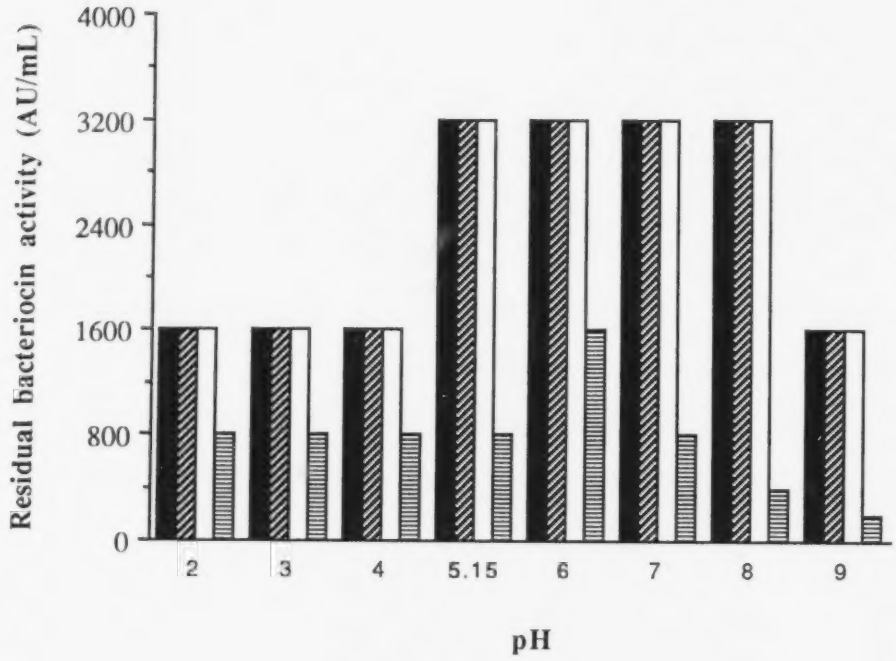

Figure 6. The effect of pH[a,d] on the thermostability of the bacteriocin produced by *Brochothrix campestris* ATCC 43754 in APT broth[b] at 25°C.

pH adjusted supernatant fluid was subjected to the various heat treatments and tested against *Carnobacterium piscicola* LV17C. The bacteriocin activity was tested immediately after the various treatments [(■) control[c]; (▨) 65°C, 30 min; (□) 100°C, 15 min; (▤) 121°C, 15 min].

a pH adjusted with 5 N HCl or 5 N NaOH

b culture grown for 17 h and centrifuged at 8000 x g (15 min) to remove cells

c unheated supernatant

d 5.15 was the pH of the supernatant after 17 h growth without pH adjustment

LV17C at 10^6 cfu/mL. At selected time intervals, samples were removed, enumerated on plates, and the optical density was monitored. Preliminary results indicate that in APT broth, brochocin-C is bacteriostatic to the indicator but bactericidal in phosphate buffer (Fig. 7). There was no change in optical density which suggests that brochocin-C does not induce cell lysis of *C. piscicola* LV17C.

Enzyme stability of brochocin-C

Partially purified brochocin-C (butanol extracted, see below) was used to determine the effect of different enzymes on the activity of the bacteriocin. Enzymes were suspended at 1 mg/mL in the appropriate buffers (Hastings *et al.*, 1991) and mixed with the bacteriocin to obtain a concentration of 6400 AU/mL. The residual activity of the bacteriocin after treatment with the enzymes was determined immediately against *C. piscicola* LV17C and is shown in Table 3. Inactivation of the bacteriocin activity by proteolytic enzymes confirms the proteinaceous nature of the compound, whereas insensitivity to lipase suggested that an essential lipid component was not part of the bacteriocin compound. The decrease in activity by treatment with lysozyme may be due to contamination by proteases or to experimental error in the interpretation of the data. Lastly, the insensitivity of the compound to catalase confirmed that the inhibitory activity was not due to hydrogen peroxide.

Stability of brochocin-C to selected solvents

Partially purified brochocin-C (butanol extracted, see below) was used to

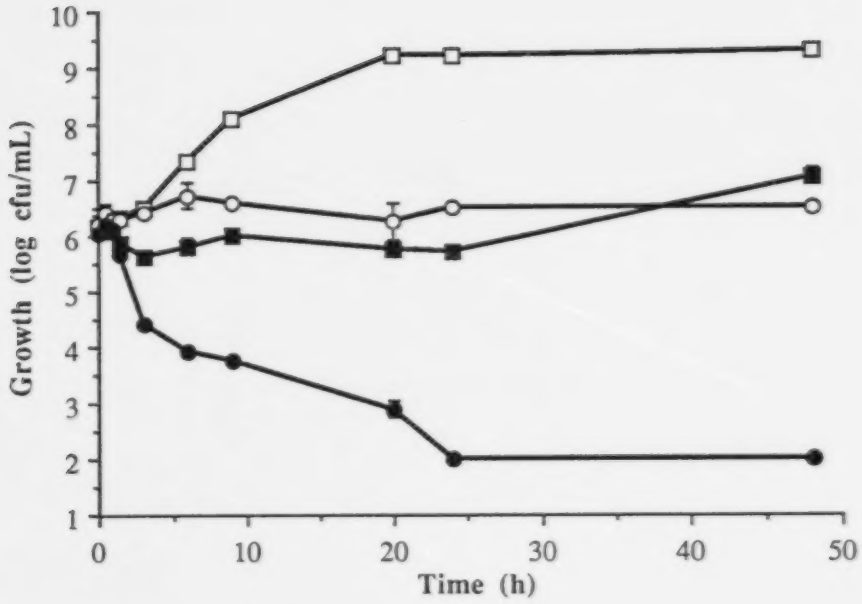

Figure 7. Inhibition of bacteriocin produced by *B. campestris* ATCC 43754 against *C. piscicola* LV17C in APT broth and in phosphate buffer (50 mM, pH 6.5).

Effect of growth of *C. piscicola* LV17C by addition of partially purified bacteriocin at 100 AU/mL in APT broth (■) and in phosphate buffer (●). Control cells without bacteriocin treatment in APT broth (□) and in phosphate buffer (o). Error bars represent standard deviation for two data points.

Table 3. Effect of enzyme[a] treatment on the activity of partially purified[b] bacteriocin produced by *B. campestris* ATCC 43754 against *C. piscicola* LV17C.

Treatment	Residual activity (AU/mL)
control[c]	6400
protease type VIII (subtilisin Carlsberg)	0
type XIV (Pronase E)	0
trypsin (type IX)	0
α-chymotrypsin	0
pepsin	0
papain (type IV)	0
lysozyme	3200
lipase (type VII)	6400
catalase	6400

a Enzymes obtained from Sigma Chemical Co. and used at 1 mg/mL in the reaction mixture.
b partially purified by extraction with butanol
c not treated with enzyme

determine the effect of selected organic solvents on the activity of the bacteriocin. These results would facilitate choosing of appropriate solvents for further purification of the bacteriocin. The bacteriocin was diluted in the appropriate solvents to a concentration of 10 AU/μL of which 10 μL was spotted on a indicator lawn of *C. piscicola* LV17C at selected time intervals and after storage at 25 and 4°C. The zones of inhibition were measured and compared with the untreated sample (Table 4). The bacteriocin was least stable in acetonitrile stored at 25°C for 24 h but it was relatively stable at 4°C for the same storage period. The bacteriocin activity was not affected by ethanol and 0.1% trifluoroacetic acid (TFA) after 6 and 24 h of storage at both test temperatures. Methanol was not as detrimental to the bacteriocin activity as acetonitrile but it was less favourable than ethanol and 0.1% TFA.

Purification of brochocin-C

A 5 litre fermentation of *B. campestris* ATCC 43754 grown in CAA medium at pH 6.7 for 22 h was used for purification of the bacteriocin. This involved extraction of the bacteriocin from the spent supernatant by butanol extraction, acetone precipitation, and size-exclusion gel chromatography (Table 5). Staining of the gel after SDS-PAGE showed only one band that correlated to the same migration distance as a zone of inhibition seen when the gel was overlayered with *C. piscicola* LV17C and thus confirmed the purity of the sample. Previous methods used in our laboratory to purify leucocin A-UAL187 (Hastings *et al.*, 1991) were unsuccessful. Incomplete binding of brochocin-C to a hydrophobic interaction

Table 4. Effect of selected organic solvents on the activity of partially purified bacteriocin produced by *B. campestris* ATCC 43754 against *C. piscicola* LV17C.

Time (h)	temp. of storage (°C)	diameters of the zones of inhibition				
		acetonitrile	ethanol	methanol	0.1% TFA	control[a]
0		20	25	22	20	22
6	25	14	22	14	19	20
24		7	25	13	23	23
6	4	15	22	19	19	20
24		16	25	19	20	21

a treated with distilled water

Table 5. Purification of brochocin-C

purification stage	vol. (mL)	activity (AU/mL)	total activity (AU)	protein conc. (mg/mL)[a]	spec.activity (AU/mg)	% recovery
supernatant	3000	3200	$9.6x10^6$	1.197	$2.67x10^3$	100
butanol extraction	100	51200	$5.12x10^6$	3.155	$1.62x10^4$	53
acetone precipitation	20	204,800	$4.096x10^6$	ND	ND	43
Sephadex G50 size exclusion	8	51,200	$4.096x10^5$	0.085	$6.02x10^5$	4.3
C_4 RP-HPLC	8	25,600	$2.048x10^5$	0.002	$1.28x10^7$	2.1

a protein concentration determined by SDS-Lowry method of Lowry *et al.* (1951) using bovine serum albumin as a standard.
ND = not determined

column resulted in large losses of activity in the initial stages. Reverse-phase high performance liquid chromatography (RP-HPLC) using acetonitrile / 0.1% TFA as the carrier solvent and a C_{18} column was also unsuccessful. The bacteriocin eluted from this column over a period of several minutes (typically in the order of 15 to 20 minutes) and showed no discernible peaks correlating to bacteriocin activity when monitored at 210 nm. This suggested that the bacteriocin was much more hydrophobic than other bacteriocins discovered thus far and was binding to the column so strongly that only high percentages of organic solvent could elute it. RP-HPLC with ethanol / 0.1% TFA as the carrier solvent and a C_4 column was successful in purifying the bacteriocin in subsequent purification trials (data not shown).

4.3 GENETIC CHARACTERIZATION OF BROCHOCIN-C.

Identification, cloning, and nucleotide sequence determination of the structural gene (*brcC*) for brochocin-C.

After purification, the N-terminal amino acid (aa) sequence of brochocin-C was determined by automated Edman degradation (Fig. 8). This revealed 47 aa residues of the peptide (Table 6a). However, the aa composition of purified brochocin-C as determined by derivitization with phenylisothiocyanate and separation by HPLC, indicated the possibility of 65 aa residues in the peptide with a calculated molecular weight of 6253.94 (Table 6b). These values were significantly different from the molecular weight of 5241.21 ± 1.22 determined by

1 23
H_2N- Y S S K D C L K D I G K G I G A G T V A G A A

24 47
G G G L A A G L G A I P G A F V A H F G V I G G -COOH

Figure 8. The N-terminal amino acid sequence of brochocin-C obtained by Edman degradation.

Table 6. Amino acid composition analysis of brochocin-C as determined by: (a) N-terminal Edman degradation of the purified protein, (b) amino acid analysis* of the purified protein, and (c) nucleotide sequencing of the structural gene.

	(a)	(b) amino acid analysis		(c)
amino acids	Edman degradation	pmol	no. of residues	nucleotide sequencing
tyrosine	1	608.8	1	1
serine	2	1812.1	3	3
lysine	3	2401.3	4	3
aspartic acid	2	3498.6	6	2
cysteine	1	67.0	0	2
leucine	3	3398.7	5	5
isoleucine	4	2840.9	5	5
glycine	14	8337.8	14	18
alanine	9	5960.8	10	11
threonine	1	1552.2	2	1
valine	3	2807.2	4	3
proline	1	1949.0	3	1
phenylalanine	2	1201.4	2	2
histidine	1	543.8	1	1
glutamic acid	0	2818.9	5	0
asparagine	0			1
total no. of residues	47		65	59
calculated molecular weight			6253.9	5245.0

* Amino acid residues were determined according to molar ratios relative to tyrosine.

mass spectrometry of the purified bacteriocin (Fig. 9). The actual aa composition of the complete peptide was later revealed by nucleotide sequencing of the structural gene (see below) and the molecular weight was calculated to be 5245, which was in good agreement with the results obtained by mass spectrometry.

A degenerate 23-mer oligonucleotide probe was synthesized [APO-1; 5'-AAAGATATTGG(ATC)AAAGG(ATC)ATTGG -3'), based on aa residues 8 to 15 of the sequence obtained by Edman degradation. The probe was dissolved in Milli-Q water, quantitated and radioactively labelled with [gamma-^{32}P]-ATP just prior to being used in hybridization experiments.

Plasmid and genomic DNA isolated from *B. campestris* was digested with *Eco*RI and blotted by the method of Southern (1975) onto a nylon membrane. Hybridization of this blot with the labelled probe at 29°C revealed unique bands in lanes corresponding to the plasmid and the genomic DNA digests, and nonspecific hybridization to a fragment of the size marker used (Fig. 10). Although all of the bands showed relatively weak hybridization, the plasmid DNA digested with *Eco*RI showed a 6-kb band that highlighted with stronger intensity compared with other bands. A second blot was done with genomic DNA digested with *Eco*RI and plasmid DNA digested with *Eco*RI, *Eco*RI - *Eco*RV, and *Eco*RI - *Hin*dIII with higher stringency conditions (44°C) for hybridization. This was too stringent and the probe did not hybridize to the membrane. No bands could be seen on the autoradiogram even after a 72 h exposure time of the film to the membrane. Rehybridization of this blot at 35 to 37°C revealed hybridization

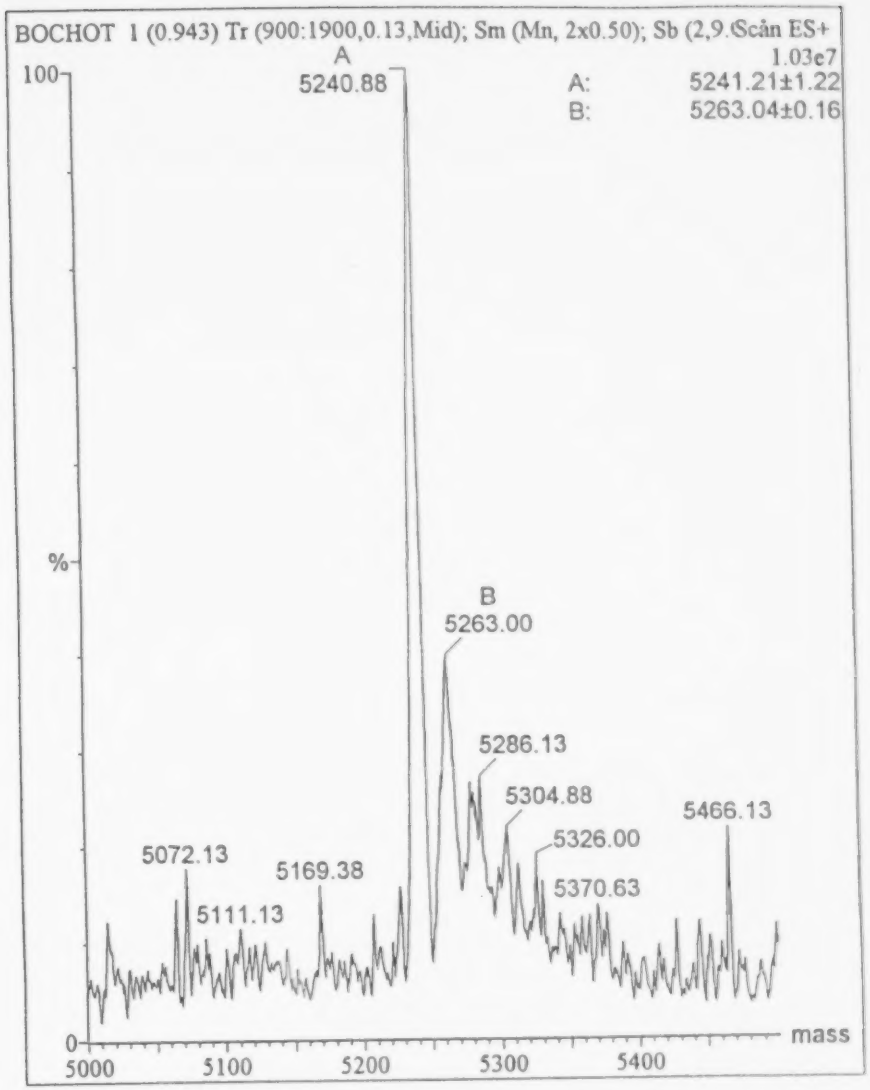

Figure 9. Electrospray fast atom bombardment mass spectrum of brochocin-C.

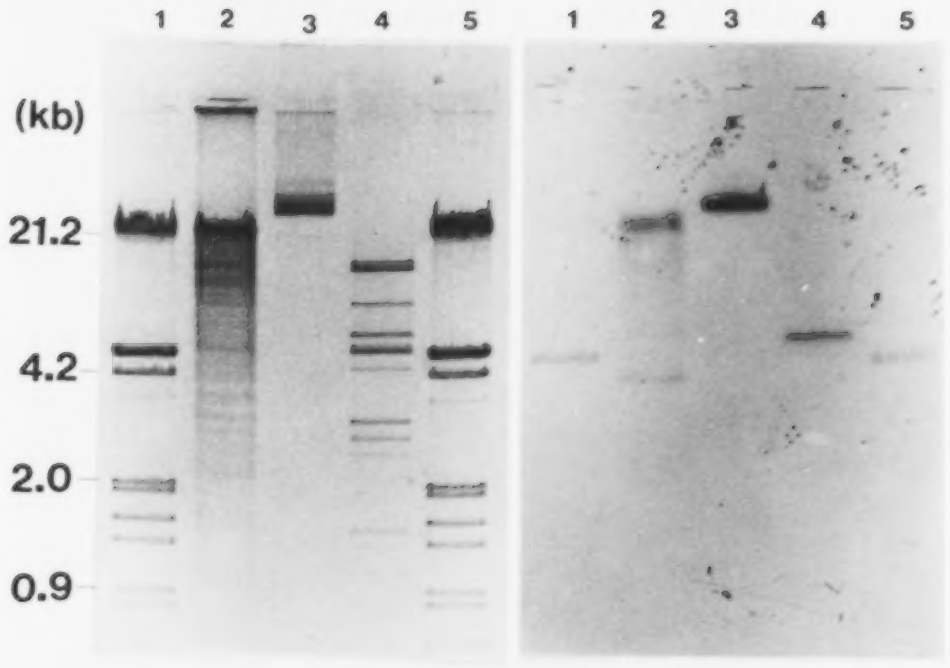

Figure 10. Agarose gel electrophoresis and Southern blot probe hybridization of restriction digests of genomic and plasmid DNA of *B. campestris* ATCC 43754. Lanes 1 and 5, *Eco*RI - *Hin*dIII digests of lambda phage size standards; lane 2, *Eco*RI digest of genomic DNA; lane 3, undigested plasmid DNA; lane 4, *Eco*RI digest of plasmid DNA. A site-specific probe (APO-1) homologous to a region within the brochocin-C structural gene was used in the hybridization.

signals with genomic DNA and the plasmid DNA, but hybridization with the fragment of plasmid DNA was stronger than with the fragment of chromosomal DNA (data not shown).

Plasmid DNA was chosen for further restriction analysis to find the smallest fragment that showed hybridization to the probe. Single restriction digestions of the plasmid DNA with selected enzymes compatible with the multiple cloning site (MCS) of pUC118 did not reveal a fragment smaller than the 6-kb *Eco*RI fragment (data not shown). Double restriction digestions of *Eco*RI with selected enzymes used in the single digests revealed a 1.5-kb *Eco*RI - *Pst*I fragment that hybridized to the probe (Fig. 11). The band intensities were stronger in this blot, in part, because the labelled oligonucleotide mixture was added directly to the hybridization solution. In previous hybridizations, labelled probe was diluted by TE buffer that was used to separate the labelled probe from unincorporated ATP during passage through a Sephadex G50 column (3 mL bed volume) before being used in the hybridization.

This *Eco*RI-*Pst*I 1.5-kb fragment was successfully cloned into the MCS of pUC118 to form the recombinant plasmid pAP4.7. Before determining the nucleotide sequence of this fragment, a blot of pAP4.7 was done to confirm that the fragment that had been cloned had homology to APO-1. Both radioactive hybridization of pAP4.7 with APO-1 and nonradioactive hybridization of *B. campestris* plasmid DNA with a 1.5-kb *Eco*RI - *Pst*I probe isolated from restriction digestion and gel electroelution of pAP4.7 (see methods, p. 37) and labelled with

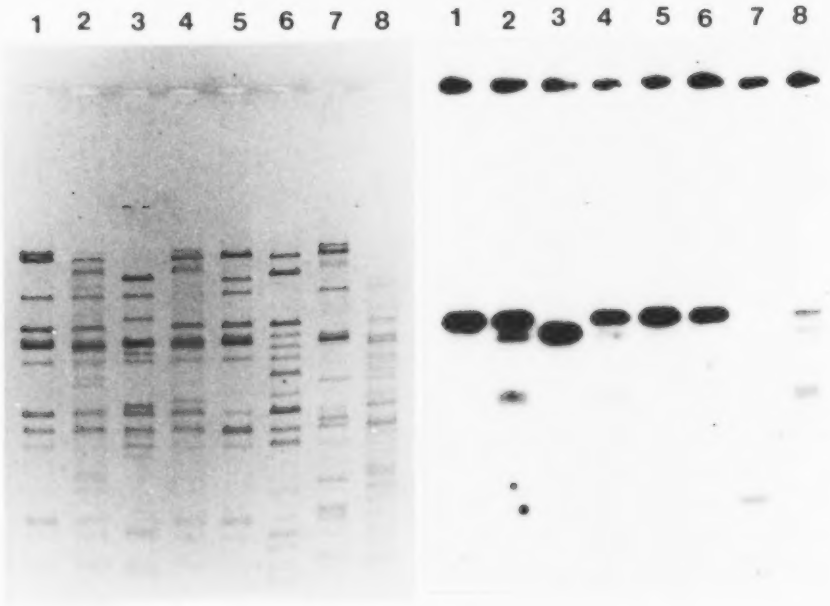

Figure 11. Agarose gel electrophoresis and Southern blot probe hybridization of double restriction digests of plasmid DNA of *B. campestris* ATCC 43754. Lane (1) *Eco*RI; (2) *Eco*RI-*Sac*I; (3) *Eco*RI-*Ava*I; (4) *Eco*RI-*Bam*HI; (5) *Eco*RI-*Bgl*II; (6) *Eco*RI-*Sal*I; (7) *Eco*RI-*Pst*I; (8) *Eco*RI-*Sph*I. The site-specific probe (APO-1) was used in the hybridization.

digoxigenin, confirmed homology to APO-1 (Fig. 12). Nucleotide sequencing of the entire fragment revealed that it was approximately 1.6 kb; however, the sequence of APO-1 was not located (data not shown). Homology searches of the APO-1 sequence with the deduced nucleotide sequence revealed 87.5% and 82.4% homology of the probe to two sites (positions 720 and 1030, respectively) within the fragment. Knowing the entire sequence of the 1.6-kb DNA fragment, a complete restriction map was constructed. This allowed selection of appropriate enzymes to pinpoint which of the two sites was responsible for the false positive signal. The 1.6-kb fragment was isolated from pAP4.7, digested with the selected enzymes, run in a 2% TBE gel and blotted onto nylon membrane (Fig. 13A). The APO-1 probe hybridized to the area at position 1030 (Fig. 13B). Analysis of this region revealed a contiguous stretch of 9 nucleotides that matched the probe (data not shown).

Genomic DNA digests were repeated using *Eco*RI. After ensuring that the digestion was complete, the DNA was run in a 0.7% TBE gel, blotted, and hybridized to the APO-1 probe (Fig. 14). Relatively low copy numbers of the hybridizing regions of both genomic and plasmid DNA could explain the low intensity of the hybridization signals. In both lanes 1 and 2, two fragments corresponding to 4.2 and 6.0 kb showed hybridization. The latter fragment was attributed to contaminating plasmid DNA because the same 6.0-kb fragment was seen in lane 3 (plasmid DNA digested with *Eco*RI). The same *Eco*RI digestion conditions, including the amount of DNA used and the time of incubation for

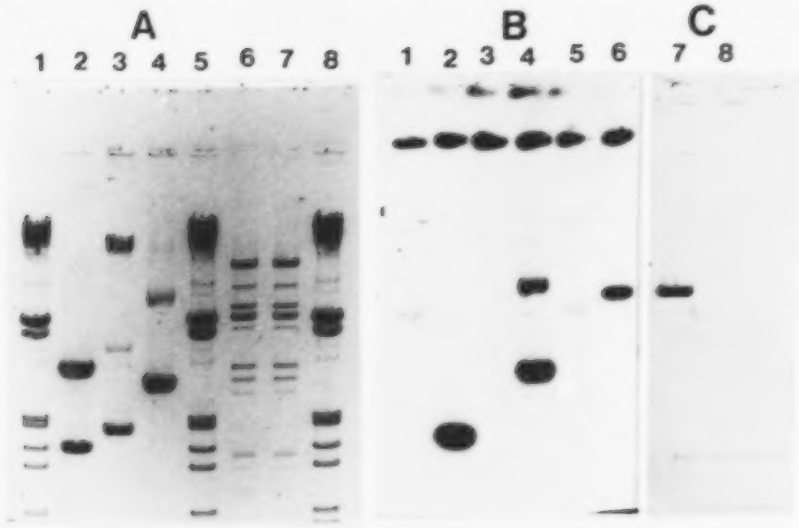

Figure 12. Agarose gel electrophoresis (A) of pAP4.7, Southern blot probe hybridization (B) with radioactively labelled APO-1, and DIG-labelled hybridization (C) with *Eco*RI-*Pst*I fragment of pAP4.7. Lanes 1, 5 and 8, *Eco*RI-*Hin*dIII digest of lambda DNA size standards; lane 2, *Eco*RI-*Pst*I digest of pAP4.7; lane 3, uncut pUC118; lane 4, uncut pAP4.7; lanes 6 and 7, *Eco*RI digest of plasmid DNA of *B. campestris* ATCC 43754.

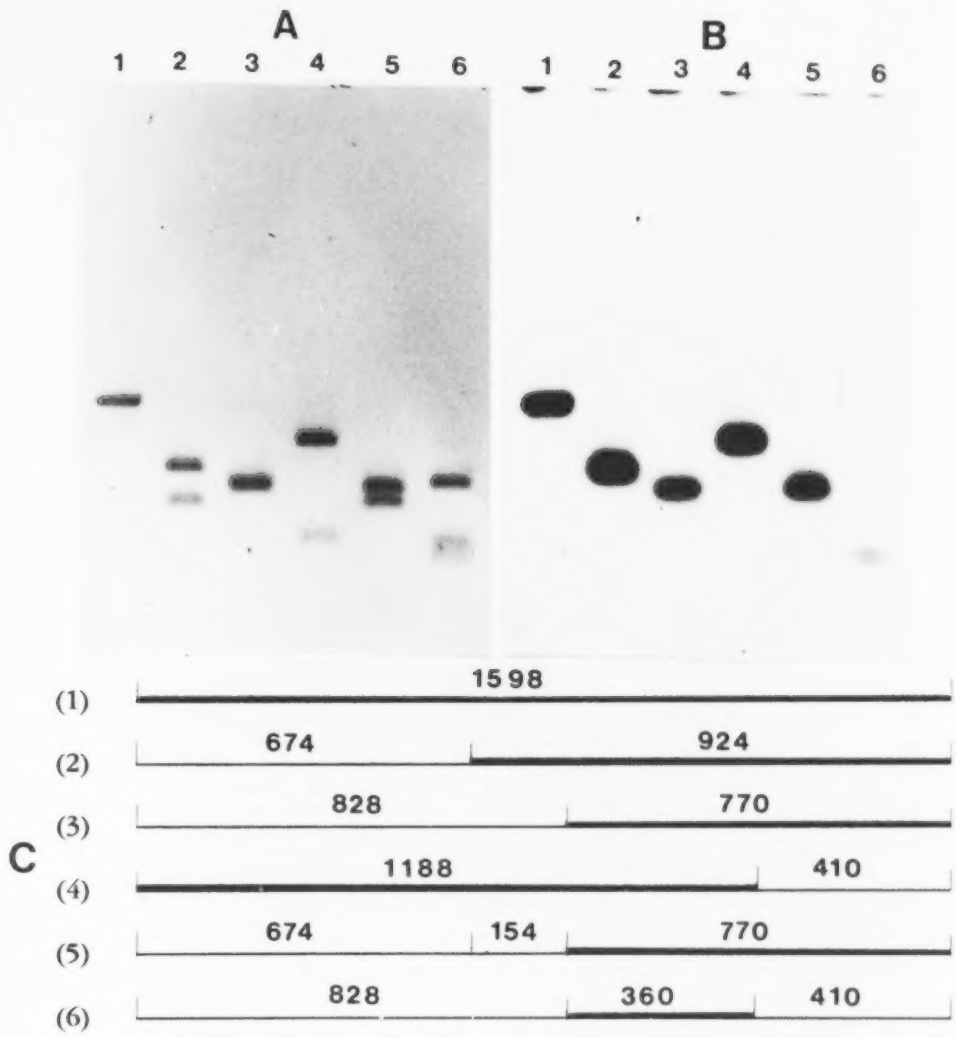

Figure 13. Identification of the hybridizing region of the *Eco*RI-*Pst*I fragment of pAP4.7 using APO-1 as probe DNA. Shown is (A) agarose gel electrophoresis of the restriction digests, (B) Southern blot probe hybridization, and (C) determination of the hybridizing region of the fragment (bold lines correspond to regions of hybridization). Lane 1, uncut fragment; lane 2, *Bst*EII; lane, 3 *Msp*I; lane 4, *Nde*I; lane 5, *Bst*EII-*Msp*I; and lane 6, *Msp*I-*Nde*I.

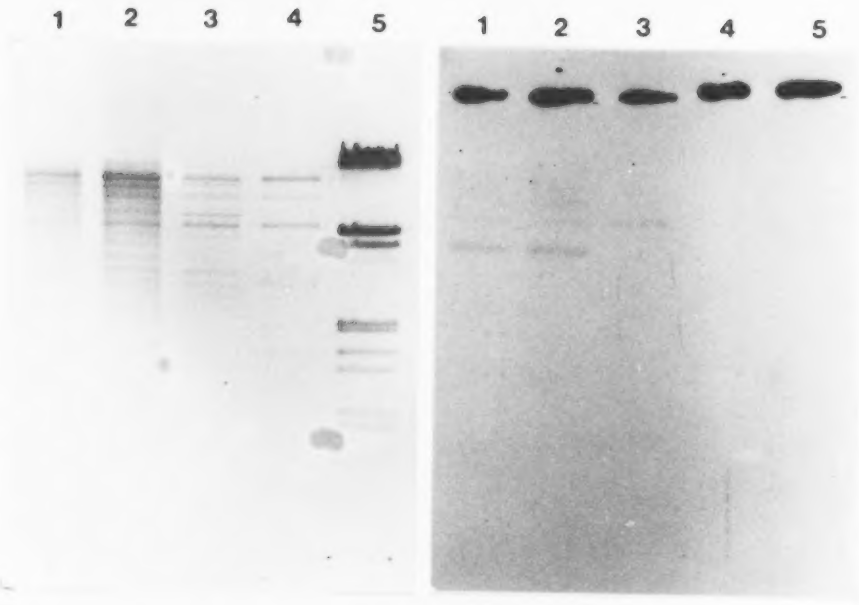

Figure 14. Agarose gel electrophoresis and Southern blot probe hybridization of genomic and plasmid DNA of *B. campestris* ATCC 43754 with APO-1. Lanes 1 and 2, *Eco*RI digest of genomic DNA; lane 3, *Eco*RI digest of plasmid DNA; lane 4, *Eco*RI-*Pst*I digest of plasmid DNA; and lane 5, *Eco*RI-*Hin*dIII digest of lambda phage DNA size standards.

restriction digestion of the DNA, was duplicated to isolate fragments of 4.2 kb from the gel by electroelution. These fragments were successfully cloned into the *Eco*RI site of pUC118. White colonies were selected and inoculated onto two large Luria-Bertani (LB) - ampicillin (200 μg/mL) plates. The colonies were grown for 8 to 12 h and blotted directly onto nylon membrane by placing it on the surface of one of two plates. The colonies on the membrane were allowed to grow for an additional 6 to 8 h on a new LB - ampicillin plate before they were lysed to release the DNA material directly onto the membrane.

Radioactive hybridization with the APO-1 probe to the membranes from the colony blots was done and revealed several clones that could contain the required insert (data not shown). These clones were identified, isolated from the master duplicate plate, and inoculated into LB - ampicillin (200 μg/mL) broth. The cultures were incubated overnight at 37°C, and their plasmid profile was determined by the small scale technique. Clones carrying an insert of approximately 4.2 kb were further screened by digestion with *Taq*I. The clones were grouped into nine representative classes based on the digestion patterns of the recombinant plasmids with this enzyme. The recombinant plasmids carrying different 4.2-kb inserts in pUC118 were digested with *Eco*RI, run in a 0.7% TBE gel, blotted, and hybridized. Only one plasmid construct (pAP7.4) showed a strong hybridization response (Fig. 15). A smaller *Pst*I fragment of approximately 1.4 kb that hybridized to APO-1 was also identified from this plasmid. The smaller fragment was successfully cloned into pUC118 to give plasmid pAP4.6.

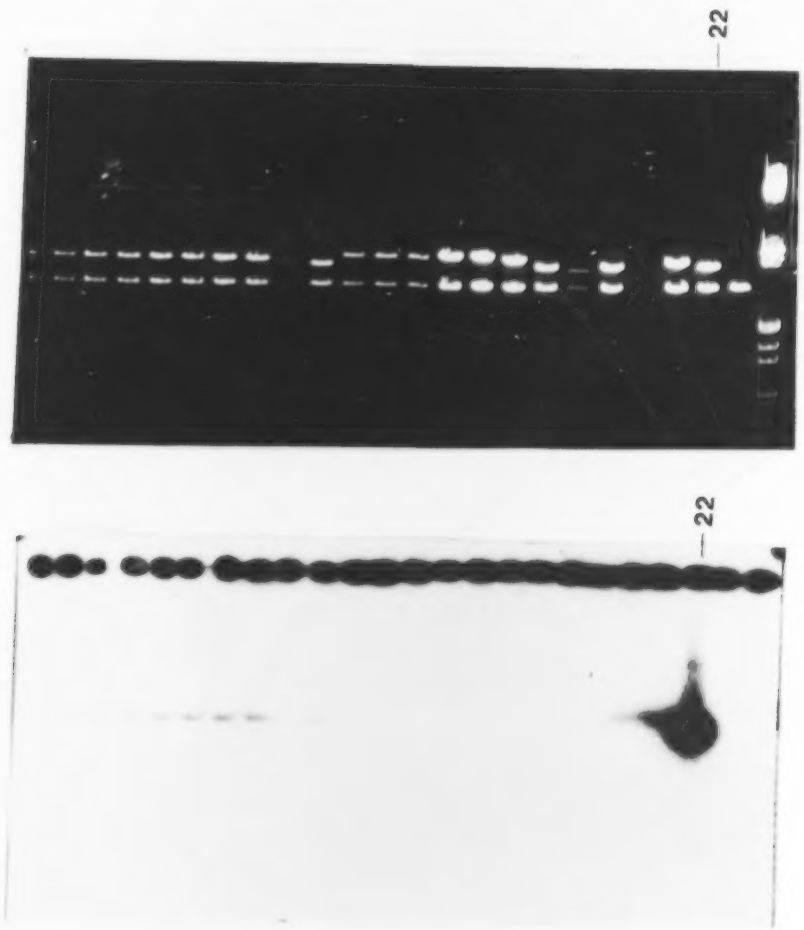

Figure 15. *Eco*RI restriction digestion of clones carrying a 4.2-kb insert in pUC118 and Southern blot probe hybridization with APO-1. Lanes 1 to 22, clones; lane 23, pUC118 cut with *Eco*RI; and lane 24, *Eco*RI-*Hin*dIII digest of lambda phage DNA size standards.

Nucleotide sequencing was initiated using pAP4.6 as the template and the universal forward and reverse primers for pUC118. This revealed part of an open reading frame (ORF1) and the sequence of APO-1. The *Pst*I site interuppted ORF1 so additional primers were made from deduced nucleotide sequences and were used to reveal the complete sequence of ORF1, the upstream and downstream regions flanking ORF1, and the complementary strand using pAP7.4 as the template (Fig. 16). The strategy used for sequencing of the fragment is shown in Fig. 17. ORF1 was identified as the structural gene (*brcC*) and consisted of 231 base pairs (bp) which encodes a primary translation product of 77 aa residues (Fig. 18). The *brcC* gene contained all of the 47 N-terminal amino acids of brochocin-C previously identified by N-terminal amino acid sequencing. The sequence of brochocin-C indicated that there would be cleavage of the polypeptide chain after the Gly - Gly residues at positions 17 and 18 during processing - maturation steps to release an 18 aa leader peptide and a 59 aa mature bacteriocin. The 18 aa N-terminal leader sequence that is cleaved after the Gly - Gly processing site has homology to other precursors of the class II LAB bacteriocins at positions -4, -7, -8, and -12 (Table 7). A putative promoter and ribosome binding site (RBS) was located upstream of the structural gene. The calculated molecular weight of the complete aa sequence of brochocin-C was 5245 (Table 6c). This was in good agreement with the value of 5241.21 ± 1.22 determined from mass spectrometry, and the difference suggests the presence of a disulphide bridge between two cysteine residues. The uncertainty of the cysteine

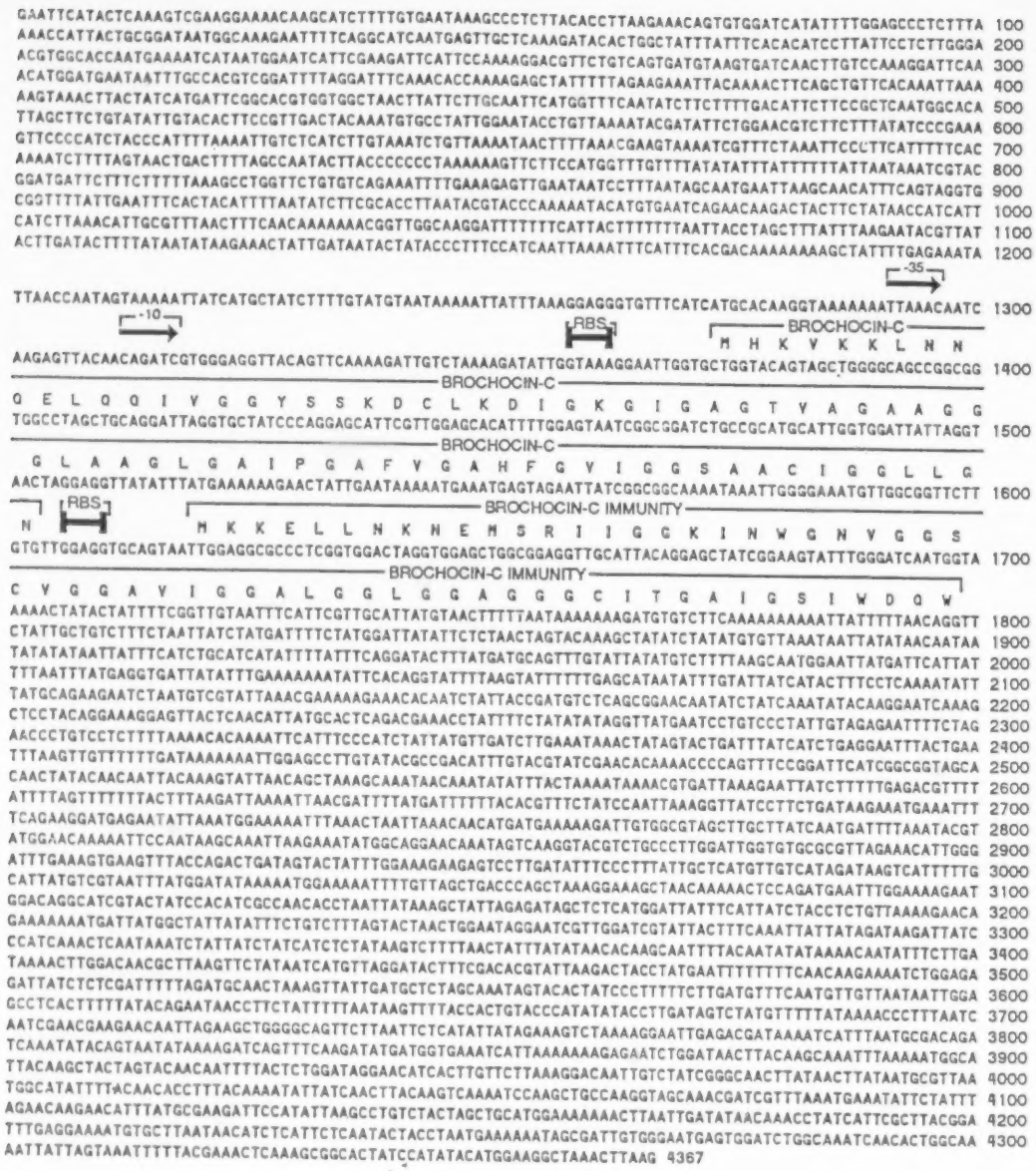

Figure 16. Single-strand DNA sequence of the 4.4-kb fragment of pAP7.4 containing the brochocin-C operon. The structural gene for brochocin-C (*brc*C) and brochocin-C immunity (*brc*I) are shown, with the translation products given below the nucleotide sequence. [RBS = ribosome binding site]

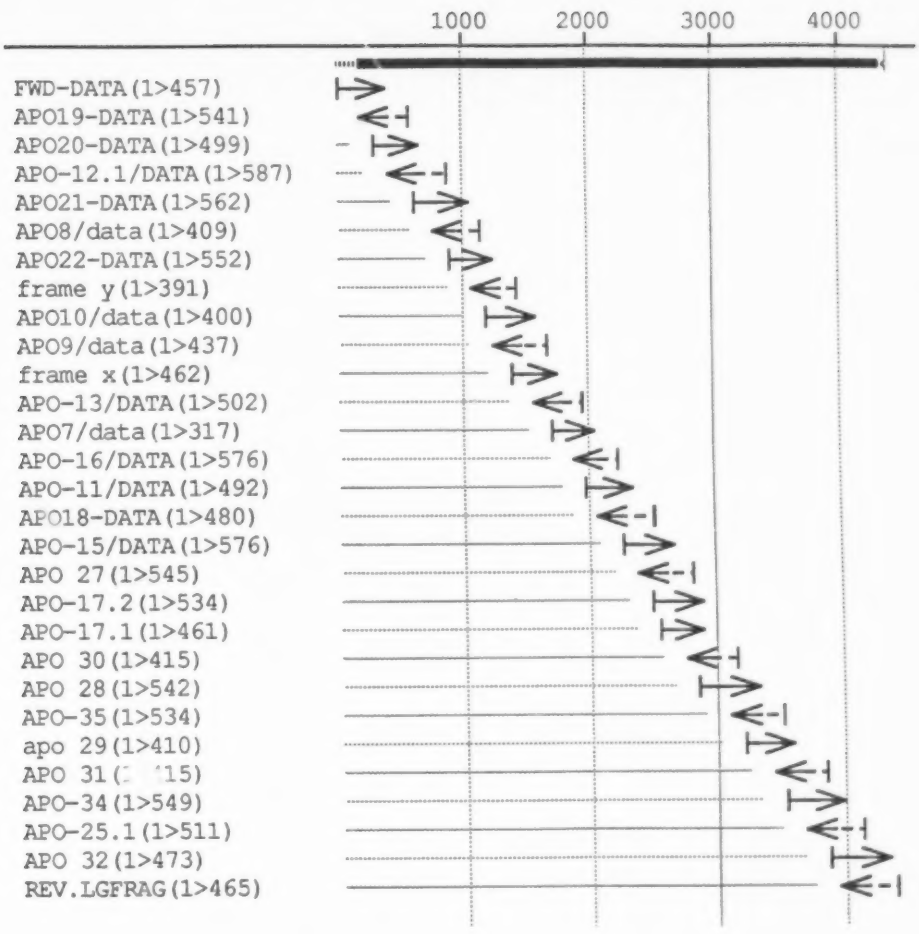

Figure 17. Stategy used for sequencing of pAP7.4.

NH_2 - M H K V K K L N N Q E L Q Q I V G G -

Y S S K D C L K D I G K G I G A G T V A G A A

G G G L A A G L G A I P G A F V G A H F G V I

G G S A A C I G G L L G N - COOH

↑

Figure 18. Amino acid sequence of prebrochocin-C.
Residues 1 - 48 (up to the ↑ symbol) were determined by Edman degradation with the exception of the underlined residue. The sequence of the leader peptide (N-terminal extension) of the prebacteriocin (small font), the underlined residue, and the residues after the arrow (↑) were interpreted from the nucleotide sequence.

Table 7. Comparison of the leader peptide sequences of the precursors of class II nonlantibiotic bacteriocins produced by lactic acid bacteria to the leader peptide sequence of the precursor of brochocin-C.

	Precursor sequence[a]																							
Bacteriocin	-24	-23	-22	-21	-20	-19	-18	-17	-16	-15	-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1
Sakacin 674/sakacin							M	E	K	F	I	E	L	S	L	K	E	V	T	A	I	T	G	G
Pediocin PA-1/AcH							M	K	K	I	E	K	L	T	E	K	E	M	A	N	I	I	G	G
Leucocin A-UAL187	M	M	N	M	K	P	T	E	S	Y	E	Q	L	D	N	S	A	L	E	Q	V	V	G	G
Sakacin A/curvacin A							M	N	N	V	K	E	L	S	M	T	E	L	Q	T	I	T	G	G
Lactacin F							M	K	Q	F	N	Y	L	S	H	K	D	L	A	V	V	V	G	G
Carnobacteriocin A/ piscicolin 61							M	N	N	V	K	E	L	S	I	K	E	M	Q	Q	V	T	G	G
Carnobacteriocin B							M	N	S	V	K	E	L	N	V	K	E	M	K	Q	L	H	G	G
Lactococcin A				M	K	N	Q	L	N	F	N	I	V	S	D	E	E	L	S	E	A	N	G	G
Lactococcin B				M	K	N	Q	L	N	F	N	I	V	S	D	E	E	L	A	E	V	N	G	G
Lactococcin M				M	K	N	Q	L	N	F	E	I	L	S	D	E	E	L	Q	G	I	N	G	G
Brochocin-C							M	H	K	V	K	K	L	N	N	Q	E	L	Q	Q	I	V	G	G
													↑				↑	↑			↑		↑	↑

[a] Homologous amino acid residues that are conserved among all of the bacteriocins listed are indicated by the arrows. Cleavage of the precursor from the mature peptide occurs after the two adjacent glycine residues at the -2 and -1 positions.

residue at position 6 from Edman degradation was verified by nucleotide sequencing of the gene and the second cysteine residue was found at position 52 of the peptide. Directly downstream of ORF1, a second open reading frame (ORF2) and RBS was identified. The translation product of this ORF was determined to be 60 aa residues and was postulated to be the immunity gene for the bacteriocin. A restriction map illustrating the position of the two ORFs is shown in Fig. 19.

A hydropathy plot was done on the 77 aa residues of prebrochocin-C by the method of Kyte-Doolittle. This revealed that residues 1 to 18 (N-terminal extension) consisted primarily of charged and hydrophilic amino acids while the mature bacteriocin (residues 19 to 77) had a charged N-terminus but otherwise it was strongly hydrophobic (Fig. 20).

4.4 EXPRESSION OF BROCHOCIN-C IN HETEROLOGOUS AND HOMOLOGOUS HOSTS

The 4.2-kb *Eco*RI fragment was successfully cloned into the shuttle vector pGKV210 to form the plasmid pAP8.6 and confirmed by nonradioactive hybridization with the 1.4-kb *Eco*RI-*Pst*I fragment of pAP4.6 (data not shown). The plasmid pAP8.6 was used to transform competent cells of *C. piscicola* LV17C and a *B. campestris* variant which had lost its ability to produce and to be immunue to brochocin-C by growing the culture in the presence of acriflavin. After electroporation, cells were plated onto APT plates containing 5 μg/mL of erythromycin and incubated for 2 to 7 d. Not many colonies grew and upon

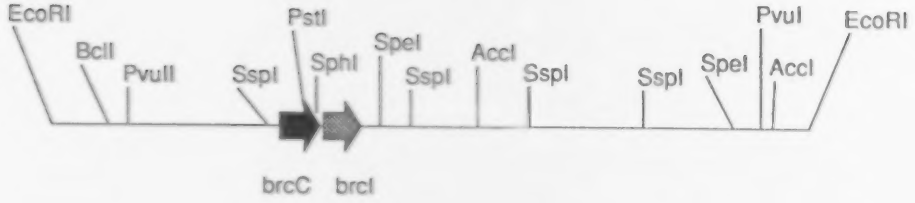

Figure 19. Restriction map of pAP7.4

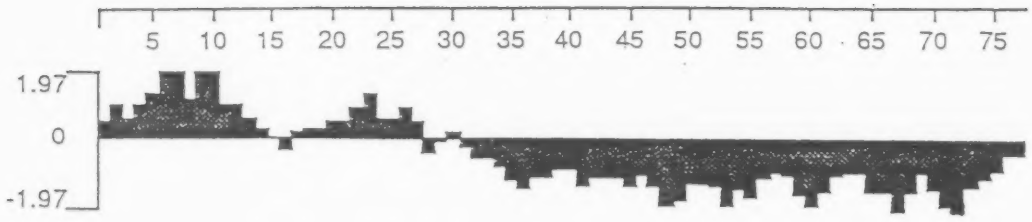

Figure 20. Hydropathy plot of the deduced amino acid sequence of prebrochocin-C. The x-axis depicts amino acid residues; the y-axis the hydropathy index. Hydrophobicity is shown as negative values; hydrophilicity as positive values.

screening, it was shown that they did not contain the recombinant plasmid. As a control, transformation was also attempted with pGKV210. This resulted in a high transformation efficiency for *C. piscicola* LV17C but not for the *B. campestris* mutant or the wild-type strain. It was concluded that *B. campestris* did not readily take up foreign plasmid DNA possibly due to problems of incompatability with the native plasmids of the organism or that pAP8.6, if it expresses brochocin-C, is somehow toxic to the cell.

5. Discussion and conclusions

This study reports the biochemical and genetic characterization of a broad spectrum, ribosomally-encoded bacteriocin peptide from the genus *Brochothrix*. To date, there are only two species identified as belonging to this genus. *B. thermosphacta* was first isolated and described in 1953, and is now well-documented as an important spoilage organism of chill stored and vacuum packaged meats (Egan and Grau, 1981; Qvist and Mukherji, 1981). The second species, *B. campestris*, has only been recently identified (Talon, 1988) and few reports have been made available in the literature about the organism (Jones, 1992; Siragusa and Nettles Cutter, 1993; Talon *et al.*, 1990). The first report of inhibitory properties associated with *B. campestris* ATCC 43754 was made by Siragusa and Nettles Cutter (1993) who, based on the inhibitory spectrum and proteinaceous nature of the compound, termed this a bacteriocin, brochocin-C. Although inhibition due to organic acids, hydrogen peroxide, and bacteriophage were ruled out, these workers did not purify the proteinaceous compound nor did they determine that it was unique by its amino acid sequence from well-characterized bacteriocins discovered thus far from lactic acid bacteria (LAB).

In our study, we found that brochocin-C in its unpurified form, had a relatively broad spectrum of inhibitory activity that included strains of *B. thermosphacta*, *Carnobacterium*, *Enterococcus*, *Listeria*, and spores of *Clostridium botulinum*. This in combination with its remarkable heat stability (65°C to 121°C) from pH 2 to 9 made brochocin-C worthwhile studying for potential application

as a natural food preservative. Inhibitory activity due to formation of hydrogen peroxide was ruled out and the proteinaceous nature of the biologically active compound reported by Siragusa and Nettles Cutter (1993) was confirmed.

Purification of the bacteriocin-like inhibitory substance was attempted using methods that have been previously shown to be successful in purifying LAB bacteriocins (e.g. leucocin A-UAL 187, Hastings *et al.*, 1991) based on their small size and hydrophobic properties. This includes hydrophobic interaction, size-exclusion chromatography, and reversed-phase high performance liquid chromatography (RP-HPLC). However, this protocol was unsuccessful in purification of brochocin-C because large losses in activity were obtained after passage through an Amberlite XAD-8 hydrophobic interaction column. The bacteriocin was not binding to the matrix efficiently because reloading of the unbound compound did not improve the recovery to any significant extent. RP-HPLC using a C_{18} column with a gradient of 0.1% TFA with acetonitrile was also not useful in the purification protocol. No discernible peaks could be associated with the biologically active fractions and spreading of the inhibitory substance over a 15 min time interval was not uncommon. This was the first clue that the compound was a very hydrophobic peptide. It may have formed large aggregates with itself and exceeded the binding capacity of the hydrophobic interaction column and in addition, because it was able to bind so tightly to a C_{18} column it was only slowly as the percentage of the organic solvent increased. This might explain why no absorbance was detected and why the activity was spread out over

such a wide range. Siragusa and Nettles Cutter (1993) reported that the compound was partially purified in their studies by ammonium sulphate fractionation. However, in our study, ammonium sulphate precipitation of the compound from spent supernatant fluids was unsuccessful. A floating pellet was encountered and this contained a significant amount of the activity. A similar phenomenon was encountered by other workers (Muriana and Klaenhammer, 1991) in the purification of lactacin F and this bacteriocin was later shown to be very hydrophobic. The purification protocol was revised to account for the strong hydrophobic properties of brochocin-C and purification was achieved with butanol extraction of the supernatant fluids, size-exclusion chromatography, and RP-HPLC on a C_4 column using 0.1% TFA with ethanol as the mobile phase. Acetonitrile was subsequently shown to have a detrimental effect on the biological activity of the compound after 24 h at 25°C but not at 4°C. Ethanol had no effect on compound stability.

The N-terminus amino acid sequence of the purified compound was determined and residues 8 to 15 were used to construct a degenerate oligonucleotide probe (APO-1). The probe sequence hybridized to endonuclease restriction digests of both plasmid and genomic DNA, but the intensity of the signal appeared to be weaker with the latter. Increasing the stringency by a slight elevation of the temperature resulted in loss of signal to both forms of genetic information. A fragment from the plasmid DNA that hybridized to the probe was cloned into pUC118 and sequenced but the structural gene for the bacteriocin was

not located. The genomic DNA fragment that hybridized to the probe was subsequently cloned, its nucleotide sequence was determined and an open reading frame (ORF) correlating to the structural gene for brochocin-C was found. The false positive signal might have been attributed to a higher copy number of plasmid DNA with a remotely familiar sequence compared with the single copy of the structural gene on the chromosome. Sequencing of the fragment carrying the structural gene also revealed a second ORF directly downstream that encoded for a 60 amino acid peptide and was postulated to be the immunity gene. This is relatively small by comparison to most immunity proteins of LAB that have been characterized to date (95 to 115 amino acids). A recently characterized bacteriocin (Worobo *et al.*, 1995) from *Carnobacterium divergens* LV13 termed divergicin A, has been shown to be a bacteriocin that does not require dedicated secretion and maturation genes, and has a postulated immunity gene of only 54 amino acids. Divergicin A has a leader peptide sequence that is cleaved during formation of the active peptide but which is unique in that it resembles that of a signal sequence. Fusion experiments by McCormick *et al.* (unpublished data) in our laboratory with the signal peptide sequence of divergicin A with the two ORFs of the brochocin-C gene cluster have indicated that the second ORF is the immunity gene. DNA sequencing of the structural gene for brochocin-C also revealed that it is synthesized as a prepeptide (presumed to be biologically inactive) and cleaved after characteristic double glycine residues at the -1 and -2 position to release the (active) mature peptide. The similarity in the location of the cleavage site for the

bacteriocin, the conservation of homologous amino acids in the leader sequence, and the fact that the length of the cleaved leader sequence is 18 amino acids indicates that brochocin-C bears remarkable homology to the class II bacteriocins of LAB. Similarities in the leader peptide sequence of brochocin-C with class II bacteriocins of LAB suggest that excretion occurs in a similar manner, i.e. by a signal sequence independent pathway with a dedicated peptidase recognizing the double glycine motif (Klaenhammer, 1993). Heterologous expression studies done by transformation of *C. piscicola* LV17 with the lactacin F operon showed that lactacin F could be concurrently produced with the carnobacteriocins of the host strain (Allison *et al.*, 1994). Therefore, the dedicated processing and secretion system for these antimicrobial peptides may be common among bacteriocin-producing strains. The fact that the class II bacteriocins usually have a narrow spectrum of activity whereas brochocin-C has a broad spectrum is remarkable. The group of bacteriocins termed lantibiotics because of their lanthionine ring structures that are formed from the dehydrated amino acids of serine and threonine, generally have a broader inhibitory spectrum. However, this clearly does not explain why brochocin-C has such a broad spectrum on inhibitory activity because unusual amino acids were not detected in amino acid determination of the purified compound.

The fact that bacteriocin is produced by a species of the genus *Brochothrix* expands the genera of Gram-positive bacteria able to produce bacteriocins. Bacteriocins have the potential to be used as food preservatives but it is necessary

to assess their applicability in a given food system. Brochocin-C may have future application as a food preservative because it is able to control growth of spoilage organisms such as *B. thermosphacta* and because it is soluble and active over a wide pH range. However, further research with this bacteriocin is necessary to assess more fully its potential in a given food system. For instance, it would be necessary to investigate whether the bacteriocin could be inactivated by proteolysis, if binding of the bacteriocin to food constituents could occur, and possible means of effectively delivering the bacteriocin to the foods. In the case of pediocin AcH, encapsulation of the bacteriocin in liposomes was shown to protect the bacteriocin against inactivation by meat constituents (Degnan and Luchansky, 1992). Furthermore, *B. campestris* is of soil origin and thus would not be easily approved for addition to meats even if it was shown to be nonpathogenic and not detrimental to the organoleptic properties of the food. Consequently, all of the genetic information necessary for production of the bacteriocin peptide needs to be transformed into an organism with food grade status, such as a lactic acid bacterium, if it is to be applied in a food system. This may also involve locating genes necessary for secretion and maturation of the peptide because these were not in close proximity to the genes responsible for production and immunity to brochocin-C.

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